# An integrative ENCODE resource for cancer: interpreting non-coding mutations and gene regulation

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## Abstract

ENCODE now comprises thousands of functional genomics data sets that may be used to investigate numerous cancer types. It is thus possible to tailor ENCODE data into a targeted resource for interpreting cancer genomes. In particular, this resource has been used to measure the impact of non-coding mutations, which constitute the overwhelming majority of somatic variants. By integrating novel assays (e.g., STARR-seq) with many epigenetic features, we can significantly refine and compactify annotations (beyond a more generic genome annotation), thereby increasing the power for recurrent-mutation detection. ENCODE functional characterization signal data, especially that related to replication timing (e.g., Repli-seq), allow us to build precise, cancer-matched background models for mutation rates that are considerably more accurate than those of previous models. ENCODE data also allow the construction of various regulatory networks that, in addition to large-scale TF ChIP-seq, incorporate data from newer assays, such as Hi-C and RNA-binding protein assays (e.g., eCLIP). In some contexts, these networks reveal how connections may be rewired during oncogenesis, as well as how such changes relate to the stem-like. More generally, these ENCODE-based networks can be used to prioritize regulators that are most associated with large-scale expression changes in cancer. Combining the networks with the refined annotations and background models, we develop a step-wise prioritization scheme for non-coding mutations. Here, we demonstrate how this can be instantiated in practice, by prioritizing non-coding mutations with significant consequences in cancer. We then successfully validated their regulatory potential via a number of small-scale validations (e.g., luciferase assays and shRNA knockdown).

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## Introduction

Large-scale functional genomics data are useful for dissecting cancer genomes, particularly for interpreting mutation and expression profiles. The initial ENCODE release in 2012, along with other targeted functional genomic data, have motivated a number of integrative studies. These data allow us to assign functional impact to non-coding mutations, which constitute the bulk of mutations in cancer genomes. For instance, Torchia *et al*. integrated various genomic and epigenetic signals to identify promising therapeutic targets in rhabdoid tumors. In addition, ENCODE data sets (especially those related to replication timing and other signals) are useful for estimating background mutation rates (BMR), which vary greatly over the genome. Lawrence *et al*. incorporated genome-wide features, such as replication timing, methylation, and expression profiles, to identify cancer drivers after BMR correction. ENCODE data are also useful for connecting non-coding elements (such as enhancers or promoters) into regulatory networks, which are pivotal for understanding cancer from a systems-biology perspective. For example, Leiserson *et al.* discovered significantly mutated subnetworks that contain well-known cancer signaling pathways in various cancer types.

The new release of ENCODE data has a number of improvements over the previous one. The novel features of the most recent data release allow us to construct EN-CODEC (a customized “companion *ENCODE* encyclopedia resource for *C*ancer”) by integrating 2,656 experiments from 14 experimental assays in 229 cell types. It comprises three parts: a background mutation rate model, compact annotations, and regulatory networks. All annotations, models, analysis results and codes are freely accessibly for users (see supplements). We detail each of these parts below and provide illustrations of how they may be used to dissect cancer genomes after combining mutation and expression profiles from large cancer cohorts such as TCGA. [JZ2MG: do we need the real URL?]

Specifically, with a much wider selection of ENCODE cell types, our ENCODE companion resource provides substantially more functional genomics data that can be better matched to particular cancer types of interest, allowing a demonstrably improved BMR model. In addition, for a number of well-known cancer cell types, it incorporates various types of novel assays (such as STARR-Seq, HiC, and ChIA-pet) with a large battery of data on histone marks to accurately define core enhancers and their target genes. Consequently, relative to generic annotations, it constructs more compact annotations in a cell type specific way to maximize statistical power in the search of mutationally burdened regions. Finally, our resource significantly extends TF regulatory networks and constructs additional networks from novel assays such as eCLIP and HiC. In a few prominent cancer types, these provide cell type-specific networks in both tumor and normal cells, which enable us to directly measure regulatory changes during the normal-to-tumor transition. Furthermore, the stem cell data in ENCODE enable us to relate such rewiring events as changes that reflect cellular states which become more or less stem-like in nature. More generally, our network can better explain cancer-specific expression patterns in tumors from resources such as TCGA, and it also helps reveal key regulators that drive large-scale tumor-to-normal expression changes.

We combined this network analysis with the compactified annotation sets and mutational burdening data (from the enhanced background model) to propose a step-wise prioritizing scheme to pinpoint key mutations associated with tumor genesis or progression. We validated the functional impact of prioritized mutations and elements using focused experiments such as shRNA-seq and luciferase assays. We emphasize such prioritization as an illustration of how new annotation sets can immediately be used to analyze existing cancer mutation data and cancer-associated gene expression.

## Multi-level data integration improves variant recurrence analysis in cancer

One of the most powerful ways of identifying key elements in cancer genomes is through mutation recurrence analysis, the objective of which is to discover regions that harbor more mutations than expected. Hence, we demonstrate how to integrate extensive ENCODE data to construct an accurate background mutation rate model in a wide range of cancer types. Accurate BMR estimation is non-trivial – the somatic mutation process can be influenced by numerous confounders (in the form of both external genomic factors and local sequence context factors), and these can result in false conclusions if not appropriately corrected10.

We address the issues associated with confounding factors in a cancer-specific manner. Specifically, we separated the whole-genome into bins (1Mb) and calculated bin-wise mutation counts under each local context category. For each category of BMR prediction, we used a negative binomial regression of the mutation counts against 475 genomic features across 229 cell types, including replication timing, chromatin accessibility, Hi-C, and expression profiles. In contrast to methods that use data from unmatched cell types10, our approach automatically selects the most relevant features, thereby providing noticeable improvements in BMR estimation (Fig 2A). For example, including matched replication timing data significantly outperforms data from the unmatched HeLa-S3 cell line. Notably, the combination of many different genomic features significantly improves the estimation accuracy in multiple cancer types (Fig 2 B). Consistent with previous results on mutation rates in breast cancer, we observed elevated mutation rates in regions with the repressive mark H3K9me3 and a reduced mutation rate in regions with the activating, enhancer-associated mark H3K27ac11-13. In addition, due to the correlated nature of genomic features across cell types, even approximate matching of a specific cancer type to a particular ENCODE cell line can still improve BMR estimation. Hence, our analyses may easily be extended to other cancer types.

 A second advantage of leveraging ENCODE data in cancer mutation analysis is provided by maximizing the statistical power of burden tests. In traditional genomic analyses, a comprehensive set of annotations (usually covering as many base pairs as possible) is considered to be beneficial. However, testing every possible nucleotide in the genome in mutation recurrence analysis noticeably reduces statistical power. Here, we aim to increase the power of burden tests by multi-level data integration from several sources. First, for a single burden test on an individual genomic element (e.g., an enhancer), focusing on a smaller, "core" region, enriched for true functional impact, would significantly improve detectability (see supplement). Hence, we trimmed the conventional annotations to key "functional territories" by using the TF-binding sites (TFBS) and the shapes of various genomic signals (see supplement). Second, repeated burden tests on a large number of elements would be subject to large penalty from multiple-testing correction.

We therefore tried to develop a minimum number of high-confidence annotations in our search for burdened regions by removing low-confidence annotations as much as possible. With a particular focus on enhancers, we started by searching for regions supported by multiple lines of evidence in the data-rich top-tier cell types. We developed a machine-learning algorithm to combine shapes of signal tracks from DNase-seq and a battery of up to 10 histone modification marks. Using a second algorithm (details in supplements), we then intersected these predictions with those of putative enhancers called from STARR-seq experiments. These experiments provide a direct, albeit noisy, readout of enhancer activity in specific cell types. Such an integrative approach enables us to define a minimal list of enhancers with as few false-positives as possible (see supplement). We also reconciled and cross-referenced our "compact annotation" with the main encyclopedia annotations (see supplement).

To increase statistical power, one final part of our compact annotation entails linking noncoding regulatory elements to protein-coding exons to form an extended gene region as a single test unit. Such a unified annotation enables joint evaluation of the mutational signals from distributed yet biologically connected genomic regions. Traditional methods for linking rely solely on the correlation of individual signals (e.g., between the activity of one histone mark at an enhancer and gene expression of neighboring genes), and these may result in inaccurate extended gene definitions. Here, we use direct experimental evidence on physical interactions from Hi-C and ChIA-PET experiments, combined with a machine learning algorithm that takes into consideration the wide variety of histone modification marks and gene expression to delineate accurate enhancer-target gene linkages.

By integrating our compact annotation sets, BMR estimates, and accurate extended gene definitions , we were able to obtain maximal power for detecting genomic regions (coding and non-coding) that are mutationally burdened (Fig 2C). For example, in the context of chronic lymphocytic leukemia (CLL), our analyses identified well-known highly mutated genes (such as *TP53* and *ATM* that have been reported from previous analyses. It also discovered genes missed by the exclusive analysis of coding regions, such as BCL6. Variants of BCL6 are known to have strong prognostic value for patient survival (Fig. 2D).

## Interpreting tumor expression profiles using ENCODE regulatory networks identifies key regulators in cancer

EN-CODEC provides detailed regulatory networks that are directly based on experimental assays, thereby making this resource ideally suited for cancer research. Specifically, for TF networks, we incorporated both distal and proximal networks by linking TFs to genes, either directly by TF-promoter binding or indirectly via TF-enhancer-gene interactions in each cell type. We then pruned these networks to include only the strongest edges using a signal shape algorithm14. In addition, we reconcile all our cell-type-specific networks to form a generalized pan-cancer network. Similarly, we also defined an RNA-binding protein (RBP) network. Compared to imputed networks derived from gene expression or motif analyses, our ENCODE TF and RBP networks were built using ChIP-seq and eCLIP experiments, which provide much more accurate regulatory linkages between functional elements.

These networks are useful for interpreting gene expression data from tumor samples. In particular, using a machine learning method, we integrated 8,202 tumor expression profiles from TCGA to systematically search for the TFs and RBPs that most strongly drive tumor-specific expression. For each patient, we test the degree to which a regulator’s activity correlates with its target's tumor-to-normal expression changes. We then calculated the percentage of patients with these relationships in each cancer type and present the overall trends for key TFs and RBPs in Fig. 3A.

As expected we found that the target genes of MYC are significantly up-regulated in numerous cancer types, which is consistent with its well-known role as an oncogenic TF15. We further validated MYC’s regulatory effects using knockdown experiments in breast cancer (Fig 3). Consistent with our predictions, the expression of MYC targets is significantly reduced after MYC knockdown in MCF-7 (Fig 3B). We then used the regulatory network to investigate how MYC works with other TFs. We first looked at MYC’s targets genes shared with a second TFs, as shown in the triplets in Fig 3C. In all cancer types, we found that the shared target genes’ expression are positively correlated with MYC, while they showed limited correlation with the second TF (as determined by partial correlation analysis).

We further investigated the exact structure of these regulatory triplets. The most common one is the well-understood feed-forward loop (FFL). In this case, MYC regulates both another TF and a common target of both MYC and that TF (Figure 3 C). Since MYC amplification is a major determinant of many cancers, understanding which TFs appear to further amplify its effects may yield insights for efforts aimed at MYC inhibition16. Most of the FFLs we observed involve well-known MYC partners such as MAX and MXL1. However, we also discovered many involving NRF1. Upon further examination, we found that that the MYC-NRF1 FFL relationships were mostly coherent, i.e., "amplifying" in nature (Fig. 3C ii). We further studied these FFLs by organizing them into logic gates, in which two TFs act as inputs and the target gene expression represents the output17. We show that most of these gates follow either an OR or MYC-always-dominant logic gate. Thus, the ENCODE regulatory network does not only identify key cancer regulators, but also demonstrates how these work in combination with other regulators.

Upon analyzing the RBP-network, we also found key regulators associated with cancer. For example, the ENCODE eCLIP profile for the RBP SUB1 has peaks enriched on the 3’UTR regions of genes, and the predicted targets of SUB1 were significantly up-regulated in many cancer types (Fig. 3C). As an RBP, SUB1 has not previously been associated with cancer, so we sought to validate its role. Knocking down of SUB1 in HepG2 cells significantly down-regulated its targets (Fig. 3D), and the decay rate of SUB1 targets is significantly lower than those of non-targets (see supplement). Moreover, we found that up-regulation of SUB1 targets is correlated with a poorer patient survival in some cancer types, such as lung cancer (Fig. 3D). These results implicate SUB1 has potentially playing an oncogenic role.

We further analyzed the overall TF regulatory network by systematically arranging it into a hierarchy (Fig 4). Here, TFs are placed in different levels such that those in the middle tend to regulate TFs at the bottom and, in turn, are more regulated by top-level TFs18. In the hierarchy, we found that the top-layer TFs are not only enriched in cancer-associated genes, but also more significantly drive differential gene expressions in tumors.

**Cell-type specific regulatory network highlights extensive rewiring events during oncogenesis**

For the top-tier cell types with numerous TF ChIP-seq experiments, our resource contains cell-type-specific regulatory networks for several cancer types, which enables direct comparison with networks built from their paired normal cell types. We proposed the concept of a “composite normal” by reconciling multiple related normal cell types (see supplement). Although the pairings (i.e., relating cancerous cell lines to specific tumors and then matching them to normal cell types) are approximate in nature, many of them have previously been widely used in the literature (see supplement). Furthermore, they leverage the extensive functional characterization assays in ENCODE to provide us with a novel opportunity to directly understand the regulatory alterations in cancer.

In particular, in "tumor-normal pairs", we measured the signed, fractional number of edges changing (which we call the “rewiring index") to study how the targets of each common TF changes over the course of oncogenic transformation. In Fig. 5A, we ranked TFs according to this index. In leukemia, well-known oncogenes (such as MYC and NRF1) were among the top edge gainers, while the well-known tumor suppressor IKZF1 is the most significant edge loser (Fig 5A). Mutations in IKZF1 serve as a hallmark of various forms of high-risk leukemia19-21. We observed a similar trend in TFs using distal, proximal, and combined networks (see details in supplement). This trend was consistent across cancers: highly rewired TFs such as BHLHE40, JUND, and MYC behaved similarly in lung, liver, and breast cancers (Fig 5).

In addition to direct TF-to-gene connections, we also measured rewiring using a more complex gene community model. The targets within the TF regulatory network were characterized by heterogeneous network modules (so called "gene communities"), which come from multiple biologically relevant genes. Instead of directly measuring the changes in a TF's targets between tumor and normal cells, we determined the changes in its gene communities via a mixed-membership model. Similar patterns to the direct rewiring were observed using this model (Fig 5A).

We next tested whether the gain or loss events from normal-to-tumor transitions result in a network that is more or less similar to that in stem cells like H1-hESC. Interestingly, the gainer TF group tends to “rewire away” from the stem cell’s regulatory network, while the loser group is more likely to rewire in such a way that it becomes more stem-like.

The majority of rewiring events were associated with noticeable gene expression and chromatin status changes, but not necessarily with mutation-induced motif loss or gain events (Fig. 5A). This is consistent with previous discoveries that most non-coding risk variants are not well-explained by the current model22. For example, JUND is a top gainer in K562. The majority of its gained targets in tumor cells demonstrate higher gene expression, stronger active and weaker repressive histone modification mark signals, yet few of its binding sites are mutated. With a few notable exceptions (see supplement), we found a similar trend for the rewiring events associated with JUND in liver cancer and, largely, for other factors in a variety of cancers. On a related note, we organized the cell-type-specific networks into cell-type-specific hierarchies, as shown in Figure 4. Specifically, in blood cancer, the more mutationally burdened TFs sit at the bottom of the hierarchy, whereas the TFs more associated with driving cancer gene expression changes tend to be at the top.

## Step-wise prioritization schemes pinpoints deleterious SNVs in cancer

Summarizing the analysis above, our companion resource consists of annotations summarized in Fig. 6 : (1) a BMR model with a matching procedure for relevant functional genomics data and a list of regions with higher-than-expected mutational burdens in a diverse selection of different cancers; (2) highly accurate, minimal and compactly defined enhancers and promotors that are defined by integrating many functional assays, including STARR-seq; (3) enhancer-target-gene linkages and extended gene neighborhoods that are obtained by integrating linkages from Hi-C and multi-histone mark and expression correlation; (4) tumor-normal differential expression, chromatin, and regulatory changes; (5) TF regulatory networks, both merged and cell-type specific, based on both distal and proximal regulation; (6) for each TF, its position in the network hierarchy and rewiring status; and (7) an analogous but less-developed network for RBPs. All the resources mentioned above have been deposited online for direct access by users (see supplements).

Collectively, these resources allow us to prioritize key features as being associated with oncogenesis. Our prioritization scheme is shown in as a workflow in Fig. 6A. We first search for key regulators that are frequently rewired, located in network hubs, sit at the top of the network hierarchy, or significantly drive expression changes in cancer. We then prioritize functional elements that are associated with these regulators, are highly mutated in tumors, or undergo large changes in gene expression, TF binding, or chromatin status. Finally, on a nucleotide level, by estimating their ability to disrupt or introduce specific binding sites (or which otherwise occur in positions under strong purifying selection), we pinpoint impactful SNVs that are further interrogated by focused functional characterization.

Using this framework, we subjected some key regulators, such as MYC and SUB1, to knockdown experiments in order to validate their regulatory effects in particular cancer contexts (Fig 3D). We also identified several candidate enhancers in noncoding regions associated with breast cancer, and validated their ability to influence transcription using luciferase assays in MCF-7. We selected key SNVs, based on mutation recurrence in breast-cancer cohorts within these enhancers that are important for controlling gene expression. Of the eight motif-disrupting SNVs that we tested, six exhibited consistent up- or down-regulation relative to the wild-type in multiple biological replicates. One particularly interesting example, illustrating the unique value of ENCODE data integration, is in an intronic region of CDH26 in chromosome 20 (Fig. 6C). The signal shapes for both histone modification and chromatin accessibility (DNase-seq) indicate its active regulatory role as an enhancer in MCF-7. This was further confirmed by STARR-seq (Fig. 5D). Hi-C and ChIA-PET data indicated that the region is within a topologically associated domain and validated a regulatory linkage to the downstream breast-cancer-associated gene SYCP223,24. We observed strong binding of many TFs in this region in MCF-7. Our motif-based analysis predicts that the particular mutation from a breast cancer patient significantly disrupts the binding affinity of several TFs, such as FOSL2, in this region (Fig. 6D). Luciferase assays demonstrated that this mutation introduces a 3.6-fold reduction in expression relative to the wild-type, indicating a strong repressive effect on this enhancer's functionality.

## Conclusion

This study highlights the value of our companion resource to the main ENCODE encyclopedia as a resource for cancer research. By integrating 2656 experiments from 14 different types of assays, we first demonstrate that we can construct an accurate BMR model for a wide range of cancers and customize non-coding annotations to maximize their power in mutational burdening calculations. We also built extensive regulatory networks from thousands of ChIP-seq and eCLIP experiments to directly study the regulatory changes associated with cancer, as well as to highlight key regulators. Finally, we leveraged this resource to provide a prioritization scheme for pinpointing key elements for follow-up experimental validation.

Our ENCODE companion resource comprises three parts: 1) compact annotations that are suitable for recurrent-mutation detection by maximizing statistical power; 2) cancer-specific BMR models with significantly increased accuracy; and 3) various regulatory networks and hierarchies for both pan-cancer and cancer-specific studies. It provides cancer-specific resources from pairing the top-tier cell lines to particular cancer types. Although the representative tumor and normal cell types and their pairings are approximate, the heterogeneous nature of cancer means that even tumor cells from a given patient usually show distinct molecular, morphological, and genetic profiles2. It is difficult to obtain a "perfect" match even from real tumor and normal tissues taken from a single patient.

Our study underscores the value of large-scale data integration, and we note that expanding the scale of our approach is straightforward. For example, a larger number of genomic features from matched cell types could result in better BMR estimation; more advanced functional characterization assays may generate further compact annotation sets, and more ChIP-seq/eCLIP experiments on additional factors would provide more detailed regulatory networks. Larger patient cohorts of expression and mutation profiles from many cancer types may be used to discover novel key features in cancer genomes. We also anticipate that an additional step may entail carrying out many assays on specific tissues and tumor samples. We demonstrate that such large-scale integration is technically feasible and provides further opportunities for future studies.

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