RADAR: An integrative framework for variant annotation and prioritization in post-transcriptional regulome for RNA binding proteins

Abstract

Dysregulation of RNA binding proteins (RBP) can cause numerous diseases but the effect of variants in their regulome has been barely investigated. Hence, we integrated 318 eCLIP, 76 RNA Bind-n-Seq, and 472 shRNA RNA-Seq experiments from the new release of the ENCODE project to deeply annotate the RBP regulome. First, we showed that around 90 percent of RBPs are enriched with rare variants in their binding sites, suggesting extensive purifying selections. We then proposed a variant impact scoring framework, RADAR, by combining RBP binding, structure, context, network, and conservation features with polymorphism data to provide a baseline impact score. Then we incorporated user-specific inputs, such as patient survival, expression, mutational profiles and prior knowledge of genes to reweight the variants to further highlight disease- and tissue-specific causal ones. Results on both germline and somatic variant datasets demonstrate that RADAR can successfully pinpoint disease-relevant variants and uncover the underlying regulation mechanism of post-transcriptional regulation.

1 Introduction and Background

Dysregulation of gene expression is a hallmark of many diseases, including cancer[1]. In recent years, the accumulation of functional characterization data on the transcription-level, such as transcriptional factor binding, chromatin accessibility, histone modification, and methylation, has brought great success to annotating and pinpointing deleterious variants. However, after (or simultaneously while) DNA has been transcribed to premature RNAs, genes also experience a series of precisely and delicately controlled processing, such as conversion to mature RNA, followed by transportation, translation, and then degradation in the cell. Dysregulation of any one of these steps may alter the final fate of gene products and result in abnormal phenotypes[4-6]. Despite its importance in regulation, the post-transcriptional regulome has been underdeveloped, partially due to its less systematic functional mapping as compared with the transcription-level regulome.

RNA binding proteins (RBPs) have been reported to play essential roles during both co- and post-transcriptional regulations[7-9]. They bind to thousands of genes in the cell through multiple processes, including splicing, cleavage and polyadenylation, RNA editing, localization, stability, and translation[10-14]. Recently, many efforts have been made to complete these post- or co-transcriptional regulomes by synthesizing public RBP binding profiles[15-18], which have greatly expanded our understanding of RBP regulation. Since 2016, the ENCODE consortium started to systematically map the post-transcriptional regulome using various types of assays on matched cell types. First, ENCODE has released large-scale enhanced CLIP (eCLIP) experiments for hundreds of RBPs[19]. It provides high-quality RBP binding profiles with strict quality control and uniform peak calling to accurately catalogue the RBP binding sites at a single nucleotide resolution. It also simultaneously performed expression quantification by RNA-Seq after knocking down various of RBPs. Also, ENCODE also performed quantitative assessment of context and structural binding specificity of many RBPs by Bind-n-Seq experiments \cite{24837674}.

In this paper, we collected the full catalogue of 318 eCLIP for 112 RBPs, 76 Bind-n-Seq, and 472 shRNA RNA-Seq experiments from ENCODE to construct a comprehensive post-transcriptional regulome. By combining

polymorphism data from large sequencing cohorts, like the 1000 Genomes Project, we demonstrated that 88 and 94 percent of RBPs showed significant enrichment of rare variants in coding and noncoding regions respectively. This strongly indicates the purifying selection of the RBP regulome. Furthermore, we proposed a top-down scheme, named RADAR (***R***N***A*** Bin***D***ing Protein regulome ***A***nnotation and p***R***ioritization), to investigate the variant impact in such regions. RADAR first combines RBP binding, structure, context, network, and conservation features with polymorphism data to quantify variant impact described by a universal baseline score. Then it allows tissue- or disease-specific inputs, such as differential expression, somatic mutation, and prior knowledge of genes, to further highlight relevant variants (Figure 1). By applying our scoring scheme on both somatic and germline variants from disease genomes, we demonstrate that RADAR is able to pinpoint disease associated variants missed by other methods. Finally, we implemented the RADAR annotation and prioritization scheme into a software for community use (radar.gersteinlab.org).

2 Results

2.1 Define RBP regulome through eCLIP data

Here we first used the binding profiles for 112 distinct RBPs from ENCODE to fully explore the human RBP regulome (Table S1). Many of these RBPs are known to play key roles in post-transcriptional regulation, including splicing, RNA localization, transportation and decay, and translation (Fig. S1). Many RBPs play more than one role in the cell.

Our defined RBP regulome covers 52.6M nucleotides, around 1.6 percent of the entire genome after duplicate and blacklist removal (Fig. 2A, details see methods). It is 1.5 and 5.9 times of the size the whole exome and lincRNAs respectively (35.3 Mbp and 89.5 Mbp). Only 53.1 percent of the RBP regulome is overlapped by the transcription-level regulatory elements, including transcription binding sites, open chromatin regions, and enhancers (Fig. S2). Unlike the transcription regulome, which has many distal elements, 55.1 percent of the RBP regulome is located in the immediate neighborhood of the exome regions, such as coding exons, 3' or 5' UTRs, and nearby introns (Fig. 2B, details see methods). Furthermore, in almost all annotation categories, we observed significantly higher PhastCons scores in the peak regions vs. the non-peak regions, providing additional evidence of their regulatory roles (Fig. 2C). In summary, the size the regulome, the limited overlapped with previous annotations, and the elevated conservation scores underscore the immediate necessity of computational efforts to annotate and prioritize variants in the RBP regulomes.

2.2 Universal features used for baseline RADAR score

2.2.1 Inference of purifying selection pressure in RBP binding sites

A large number of literature has pointed out that enrichment of rare variants indicates purifying selection in functional regions in human genomes[34-36]. Hence, we inferred the purifying selection pressure on the binding sites of each RBP by integrating population-level polymorphism data from large cohorts, e.g. the 1000 Genomes Project cite{26432245, 23128226}. GC percentage usually confounds the purifying selection pressure inference because it causes read coverage variations, a sensitive parameter in the downstream variant calling process [37,38]. Hence, we first calculated the fraction of rare variants (derived allele frequency (DAF) less than 0.5%) within each RBP's binding site, and compared it with those from regions with similar GC content as a background (see details in methods). In total, 88.4 percent of the RBPs (99 out of 112) show elevated rare variant fraction in coding regions compared to those of the background regions after GC correction (Fig. 3A). Similarly, in the noncoding regions of the binding sites, 93.8 percent of RBPs (105 out of 112) exhibit an enrichment of rare variants. This observation convincingly demonstrates the accuracy of our RBP regulome definition. (Table S2).

Some well characterized disease-causing RNA binding proteins are among the top RBPs with larger difference of rare variants fraction when comparing to the background regions. For example, the well-known oncogene XRN2, which binds to the 3' end of transcripts to degrade aberrantly transcribed isoforms, showed significant enrichment of rare variants in its binding sites[39]. Specifically, it demonstrates 12.7% and 10.3% more rare variants in coding and

noncoding regions (adjusted P values are 1.89×10^{-9} and 2.85×10^{-118} for one sided binomial tests)[40]. Hence, we used the enrichment of rare variants as a feature to infer the selection pressure in RBP binding sites to weight the variants in such regulator regions (details see methods).

2.2.2 highlighting variants in RBP binding hotpots

It has been reported that genes within network hubs usually exhibit greater enrichment of rare variants—a sign of strong purifying selection pressure[34,35,44]. Similarly, we suspect that RBP binding hot spots may demonstrate similar characteristics because once mutated, they may introduce larger regulation alterations. To test this hypothesis, we separated the regulome based on the number of associated RBPs. The majority (62 percent) of the regulome regions are associated with only 1 RBP (Fig. 3B and Fig. S4). As the number of RBPs increased, we observed an clear trend of higher rare variant enrichment. For instance, in the noncoding regions, regions with at least 5 and 10 RBPs exhibited 2.2 and 13.4 percent more rare variants compared to the whole genome average (top 5 and 1 percent, Fig 3C). This observation significantly supports our hypothesis that the RNA regulome hotspots are under stronger selection pressure, and hence should be given high priority when evaluating the functional impacts of mutations.

2.2.3 Motif gain/loss analysis as a feature for nucleotide impact

Mutations that change the binding affinity of RBPs may change RBP regulation via loss-of function effect. To quantify the impact of such effects, we used the difference of position weight matrix (PWM) or position probability matrix (PPM) scores of the mutant allele against the reference allele. RADAR consists of two sources of motifs. It has been reported that many of the RBPs' binding events *in vivo* can be captured by binding preferences *in vitro*. Hence, we first incorporated motifs reported by RNA Bind-n-Seq experiments from ENCODE (17 RBPs matched with eCLIP). We also used the same scheme on *de novo* motif discovered directly from the binding peaks for all 112 binding proteins by searching for enriched k-mers using the default settings in DREME (details see methods). The highest motif score from the two sources are used for each single nucleotide.

2.2.4 Structure and sequence conservations

RNA secondary structures have been reported to affect every step of the protein expression and RNA stability \cite{24821474}. We incorporated structure features predicted by Evofold, which uses phylogenetic stochastic context-free grammars to identify functional RNAs encoded in the human genome that are deeply conserved across species \cite{16628248}. We found after intersecting with Evofold predictions, the RBR regions show significantly larger enrichment of rare variants. Hence, the entropy of variants falling into such interaction regions are used to boost their impact effect.

Besides, cross species sequence conservation has also been widely used as an important feature to discover regions with biological functions. For example, The Genomic Evolutionary Rate Profiling (GERP) score was developed to identify nucleotide level evolutional constraints by mapping human genome to other species. We used GERP score in our baseline RADAR framework to detect potentially deleterious mutations in the RBP regulome.

2.2.6 Highlight differentially expressed genes after RBP knockdown

Expression profiling by RNA-seq before and after shRNA mediated RBP depletion from ENCODE can help infer the gene expression changes after RBP knockdown. For each such gene-RBP association, if a variant is located nearby to such genes and happens to disrupt the associated RBP binding by motif breaking events, we used the entropy value of such events to add to the variant's baseline score.

2.3 User-specific features to reweight variant impact

2.3.1 Use expression/survival profiles to prioritize key regulators

In many diseases, a few key regulators are usually associated with disease progression and variants that affect their regulation should be highly ranked during the prioritization process. Hence, with additional disease-specific expression or survival profiles from the user, we tried to find such key regulators by combining the RBP regulatory network information. Specifically, we first constructed the RBP network from the eCLIP profiles and defined the differential expression of disease and normal cell types. Then we used a regression based to quantify the RBPs

regulator powers as its association with expression changes. We applied this approach on 19 cancer types from TCGA and the regulatory power of the RBPs are given in Fig. 4. We found that among many of the RBPs with larger regulatory power have been reported as cancer associated genes (with * in Fig. 4B, Table S3). Interestingly, the regulatory power of two key genes PPIL4 and SUB1 were found to be significantly associated with patient survival (Fig. 4C). In our RADAR framework, we further highlight variants that are associated with significant regulators in their corresponding cancer types.

2.3.2 Prior knowledge of target genes

Variant Prioritization can be largely improved if the function of its associated genes is known. For example, many databases have numerated hundreds of cancer-associated genes that are known to play critical rules in cancer. Cell proliferation and DNA repair related genes are also important for cancer research. For other disease, many GWAS studies have good interpretation of risk genes. In addition, genes undergo significant expression or epigenetic changes are largely cell-type specific and be can used to highlight more relevant variants. Hence our RADAR frame work allows users to input their disease specific genes of interest to further prune the candidate variant list.

2.3.2 Somatic variant recurrence

Variant recurrence has been widely used as an important sign of purifying selection and regions with more than expected mutations are often considered as disease driving events. Given the somatic mutation from a large cohort of patients, we first defined a local background mutation rate to evaluate the mutation burden in each RBP peaks. Variants that are associated with burdened elements are given higher priority in the prioritization scheme.

2.4 RADAR weighted scoring scheme to prioritize variants

By integrating the universal and user-specific information mentioned above, we proposed an entropy based scoring scheme to investigate the functional impacts of variants specific to post transcriptional regulation (Fig. 1 and Fig. S5). First, we added up the (weighted) entropy score of variants for all universal features, which include rare variant enrichment, binding hotspot, structure, motif, and conservations. Then depending on the user inputs, we further up weight mutations that falls into the key RBP binding sites, nearby genes of interest, or within elements with more than expected variants.

Table 1. Features used by RADAR

Category	Feature	Source	Scoring Scheme
Universal	Selection pressure	eCLIP	Weighted-entropy
	Binding hotspots	eCLIP	Weighted-entropy
	RBP-gene association	shRNA RNA-seq	Entropy
	Motif disruption	Bind-n-Seq	Weighted-entropy
		DREME	
	Structure sensitivity	Evofold	Entropy
	Conservation	Gerp	Entropy
User-specific	RBP regulatory power	Survival	Entropy
		Expression	
	Key genes	Prior knowledge	Entropy
	Mutation Recurrence	Mutation profiles	Entropy

2.5 Application on pathological germline variants

Due to the lack of disease information in pooled disease study, we calculated baseline RADAR score on all pathological variants from HGMD. We used the 1000 genomes (1kg) variants, as the background to compare the distribution of scores. As expected, the HGMD variants are scored significantly higher than somatic mutations (Fig. S6). For example, the mean RADAR score for HGMD variants is 0.445, while it is only 0.044 for 1kg variants (P value <2.2e-16 for two sided Wilcoxon test). We further compared RADAR scores of HGMD variants with other methods (Table S6). Specifically, we found 992 HGMD variants that are explained by only our methods and 29.6%

of them are noncoding variants that are located in the nearby intron, 5'UTR, and 3'UTR (and their extended regions). An example of such variants is given in Fig 5. It is located 28 bp away from the acceptor site of exon 3 in TP53. eCLIP experiments showed strong binding evidence in 7 RBPs, most of which are splicing factors. The co-binding of these above mentioned splicing factors strongly indicate this is key splicing regulatory site. Specifically, this A to T mutation strongly disrupts the binding motif of SF3B4, increasing the possibility of splicing alteration effects. Our finding is not reflected in previous methods for variant prioritization.

2.6 Application on somatic variants in cancer

2.6.1 Somatic variants associated with COSMIC genes and recurrence

We applied our scheme to evaluate the deleteriousness of somatic variants from public datasets. Due to the lack of golden standard, we evaluate our results from two aspects. First, hundreds of cancer-associated gene are known to play essential roles through various pathways[47,48]. Hence, in general, variants associated with these genes are supposed to have a higher functional impact compared to others[34]. To test this hypothesis, we first associated each variant with a gene by the shortest distance according to Gencode v19 annotation. We found that in all four cancer types we tested, including the breast, liver, lung, and prostate cancer, variants associated with cancer associated genes showed significantly enrichment in variants with larger RNA level functional impact (Fig. S7). For example, we found a 3.27 and 3.36-fold increase in high impact variants at a threshold level of 2.5 and 3 respectively in breast cancer patients ($P < 2.2e-16$, single sided Wilcoxon). This pattern is consistent in all four cancer types we investigated (Fig S7).

In addition, because variant recurrence is considered a sign of functionality and may indicate association with cancer[34-36], we also compared the variants' score distribution from RNA binding peaks with or without recurrence. Specifically, we separated the peaks with variants from more than one sample from those that are mutated in only one sample and compared the percentage of higher impact scores. We found that in most cancer types, elements with recurrent variants are associated with a larger fraction of high impact mutations. For example, in Breast cancer, recurrent elements demonstrated a factor of 1.20, 1.55, and 1.77-fold enrichment of high impact variants with RADAR greater than 1.5, 2.5, and 3.0 respectively, resulting in a P value at 1.71e-19 from one-sided Wilcoxon test.

2.6.2 A case study on breast cancer patients

We applied our method on a set of breast cancer somatic variants from 963 patients released by Alexandrov *et al*[50] and used COSMIC genes, expression and mutational profiles as additional features. In total around 3 percent out of the 68k variants was evaluated to alter post-transcriptional regulations to some degree. Specifically, 169 out of the 501 highly ranked variants only reported by our tool are located in the noncoding regions, with 15, 28, and 24 are from nearby introns, 5' UTR and 3' UTR regions, respectively (Fig. S8). For the intronic one, we find that such variants usually bind within 30 bp of the splice sites and break the motifs of many splicing factor binding sites. For the 3' UTR regions, variants reported only by RADAR are within the binding peaks of Cleavage Stimulation Factor binding sites, strongly indicative of a role in the polyadenylation of pre-mRNAs. The discovery of such meaningful results indicates the ability of RADAR to differentiate deleterious mutations that disrupt post-transcriptional regulations.

3 Discussion

In this paper, we integrated the full catalogue of eCLIP, Bind-n-Seq, and shRNA RNA-Seq experiments from ENCODE to build the RNA regulome for post-transcriptional regulations. Our defined RBP regulome covers a larger fraction of the genome compared to that of the whole exome region and only showed some overlap with previous transcription level annotations. RBP peak regions are more conserved than the non-peak regions under the same annotation category and the majority of RBPs demonstrate significant enrichment in rare variants. These two sources of evidence consistently showed that the RBP regulome is under strong purifying selection pressure suggesting the importance of the regulome in carrying out important biological functions. It signifies the necessity of computational tools to annotate and prioritize variants in the RBP regulome, which was previously under investigated.

By integrating a variety of regulator, element, and nucleotide level features, we proposed an entropy based scoring frame RADAR to investigate impacts of somatic and germline variants. RADAR contains two parts in the variant prioritization framework. First, by incorporating eCLIP, Bind-n-Seq, shRNA RNA-seq experiments with conservation and structure features, we built a pre-defined data context to quantify the baseline variant impact score. It is suitable for multiple disease analysis or cases where no other prior information can be used. We applied this score on HGMD pathological variants and highlighted many candidates that are solely discovered by RADAR with detailed explanation of the underlying disease cause mechanism. On top of the baseline score, RADAR also allow user-specific inputs such as prior gene knowledge, patient expression, mutation and survival profiles for a re-weighting process to highlight relevant variants in a disease specific manner. We showed an example of breast cancer variant prioritization and score re-weighting scheme by user inputs in the well-known tumor suppressor gene TP53. Also results from somatic variants from several cancer type show that RADAR is able to identify relevant variants.

In summary, we believe that RADAR can serve as a useful tool to annotate and prioritize the post-transcriptional regulomes for RBPs, which has not been covered by most of the current variant functional impact interpretation tools. It is also able to provide additional layers of information to current gene regulomes. More importantly, the RADAR scoring scheme can be used in conjunction with some of the current transcriptional variant functional evaluation tools, such as Funseq, to add independent information to jointly evaluate variant impacts. With the fast expanding collection of binding profiles of more RBPs from more cell types, we envision that it can more extensively tackle the functional consequence of mutations from both somatic and germline genomes.

4 Methods

4.1 eCLIP Data Processing and Quality Control

eCLIP is an enhanced version of the crosslinking and immunoprecipitation (CLIP) assay, and is used to identify the binding sites of RNA binding proteins (RBPs). We collected all available eCLIP experiments from the ENCODE data portal (encodeprojects.org). There were 178 experiments from K562 and 140 experiments from HepG2 cell lines, totaling 318 eCLIP experiments from all available ENCODE cell lines (released and processed by July 2017). These experiments targeted 112 unique RBP profiles. eCLIP data was processed per ENCODE 3 uniform data processing pipeline. The eCLIP peak calling method and processing pipeline were developed by the laboratory of Gene Yeo at the University of California, San Diego (https://github.com/YeoLab/clipper, CLIP-seq cluster-identification algorithm on PMID: 24213538). For each peak, the enrichment significance was calculated against a paired input, and we filtered those peaks with a significance flag of 1000. We ultimately used the recommended cutoff of the significance, which was -log10(P-value) >= 3 and log2(fold_enrichment) >= 3.

4.2 Annotation

RNA binding proteins bind along the genome in a variety of contexts. Using eCLIP data, we can synthesize a genomic landscape of where RBPs bind. Raw peak signals from eCLIP data are translated into binding sites, using a peak caller specialized for eCLIP data. Generally, these RBPs having binding sites that correspond to about 150 bp, with many RBPs having well over 10,000 binding sites. Binding site locations containing blacklisted regions are removed. These include regions on the genome with low sequencing depth or coverage or […]. Despite filtering these blacklisted regions, over 99% of the binding locations are preserved. While the total number of base pairs corresponding to binding sites translates to a large number, compared to the scale of the genome it is still minute. Therefore, we annotate the genome, indicating at each position the set of RBPs that bind. This annotation set is known as the contextual annotations.

In addition to contextually annotating the genome with the preferential binding of RBPs, we also include a functional annotation – whether a specific position falls in the coding or noncoding region of the genome. The coding region consists of only the exons of protein coding genes. The noncoding region is further divided into 3'UTR, 5'UTR, 3'UTR extended, 5'UTR extended, and nearby intron regions. Coding and UTR annotations are retrieved from Gencode and UCSC, respectively. 3'UTR and 5'UTR extended regions consist of the 1000 base pairs downstream of the 3'UTR and 5'UTR regions, respectively. The nearby intron regions consist of the 100bp regions adjacent to each exon. While each of these region types are generally distinct, overlap is a possibility. Therefore, a hierarchy of which annotation takes precedence when annotation types overlap is established, from highest priority to lowest: coding,

3'UTR, 5'UTR, 3'UTR extended, 5'UTR extended, and nearby intron. Regions of the genome not classified by these annotations are labeled as "other" and may refer to other noncoding elements or blacklisted elements.

4.3 Inference of negative selection pressure from population genetics data

4.3.1 Using rare derived allele frequency as a metric for negative selection pressure

It is useful to understand the negative selection pressure associated with particular regions or locations of the genome. In order to infer the negative selection, we make use of germline variants from the 1000 Genomes Project. These germline variants consist of both common and rare variants. These variants are then classified into coding and noncoding variants. Coding variants fall in regions annotated as coding, while noncoding variants fall in regions annotated as noncoding Section (4.2). Noncoding variants are not further classified into noncoding element subgroups in order to maintain a large sample size for optimal statistical power in inferring negative selection pressure. The metric we use to represent negative selection pressure is the rare derived allele frequency (rare DAF). For a given region, i, containing rare variants r_i and common variants c_i, the rare DAF is defined to be

Rare DAF = r_i / (r_i + c_i)

Since we have further categorized both rare and common variants as coding and noncoding, we can obtain a coding and noncoding rare DAF for a given region as well. Finally, we take the rare DAF value and divide it by the GC content corrected genome average (Section 4.3.2) in order to obtain a ratio. Regions with rare DAF ratios larger than 1 suggest an above average negative selection pressure.

4.3.2 Rare DAF is confounded by GC content

Although negative selection pressure can be inferred from metrics such as rare DAF, it is not always accurate. In particular, the rare DAF of a region is severely confounded by its GC content. In order to correct for this bias, we first bin the genome into 500 base pair bins. Next, we estimate the average GC content within these 500 base pair bins, which can range from 0% to 100%. We then group bins with similar GC content. Specifically, we establish 40 groups, using 2 percent intervals from 20 to 80 percent GC. Bins containing 0-20 and 80-100 percent GC content are ignored due to limited observations in these groups. For each of the 40 groups of 2% GC intervals, we associate a set of 500 base pair bins. Each of these sets are taken together to form a region, i, and the rare DAF is calculated. For each of the 40 regions, i, we obtain a rare DAF value, forming a discrete relationship between rare DAF and GC content. Using these discrete points, we fit a Gaussian kernel smoother with bandwidth of 10, resulting in a smoothed function between rare DAF and GC. This function serves as a way to estimate the genomic rare DAF given the GC content.

4.3.3 Negative selection pressure of RBP specific binding sites

We directly apply the method of determining a corrected rare DAF ratio to binding regions for a given RBP. The GC content of all binding sites for an RBP is estimated (from a genomic bigwig file), and using the derived smooth function between rare DAF and GC, a coding and noncoding rare DAF ratio is determined. For any given RBP a rare DAF ratio is used to measure the relative selection pressure of an RBP.

4.4 Co-binding and Hotness (need to brainstorm another title)

A natural extension to annotating locations based on the set of RBPs that preferentially bind, is to include the annotation of how many RBPs bind. The value associated with the number of RBPs that bind to a position is termed the "hotness". Regions with more RBPs binding are deemed to be more "hot" than locations with fewer RBPs binding. We hypothesize that the hotness of a region and the selection pressure of the region demonstrate a positive relationship.

To determine the actual relationship, we annotate the genome with hotness on a base pair resolution. For both noncoding and coding regions, we estimate the selection pressure using rare DAF ratio from germline variants within all regions showing equal to or more extreme hotness for any given hotness. The rare DAF ratio is found by taking the rare DAF and dividing by the corrected rare DAF, derived from evaluating the GC for regions with the same hotness and predicting the genomic rare DAF average (4.3.2). We show a cumulative relationship between rare DAF and hotness, with a generally increasing trend. When the hotness increases past 10 however, the lack of observations results in difficulty in producing a reliable rare DAF. Therefore, we cutoff the measure of rare DAF at a maximum hotness of 10, corresponding to the top 1% of the data. Furthermore, regions with hotness less than 5% of the data, equal to a hotness of less than 5, are deemed to not be hot, and are automatically given a 0 value in rare DAF ratio. The resulting discrete function is smoothed from hotness of 5 to 10. The function steps from 0 (from hotness of 1 to 4) to the rare DAF ratio at 5, and also maintains a constant rare DAF ratio for hotness values over 10 by rounding them down to 10.
Many RBPs bind in similar locations across the genome, and this is measured by their co-binding percent. The co-binding between two RBPs, A and B, is defined to be the maximum ratio between the peaks that intersect between A and B and the total number of peaks for A or B. Intersection is defined for greater than or equal to one base pair. Here, the maximum is taken in order to allow for a symmetric matrix in plotting a co-binding heatmap, resulting in only a unique possible result for clustering RBPs by similarity of co-binding. Using the co-binding ratio values between pairwise RBPs, a symmetric matrix is constructed and clustering is performed. The R function pvrect in package pvclust is used for clustering with an alpha value of 0.02 instead of 0.05 in order to avoid clusters with large numbers of RBPs (>6). The resulting clusters of RBPs with significance were found to follow patterns of functional co-binding found in literature.

4.5 Motif analysis

4.5.1 De novo discovery

RBP motifs were found using DREME software (Version 4.12.0, http://meme-suite.org/tools/dreme, Timothy L. Bailey, "DREME: Motif discovery in transcription factor ChIP-seq data", Bioinformatics, 27(12):1653-1659, 2011.). De novo motif was called on a collection of significant eCLIP peaks.

4.5.2 Evaluating Motif Disruption with MotifTools

To evaluate the functional importance of RNA-binding sites, we surveyed mutational impact on RBP motifs. We called potential RBP motifs on high-confidence RBP peaks and evaluated motif disruption power of each variant using a germline variant set (1000 Genomes Project, a somatic variant set (30 types of cancer somatic SNVs, Alexandrov et al., Nature 2013), and HGMD (version 2015 *** please confirm the version ***). Motif breaking power, which we labeled as D-score (D stands for disruptive-ness or deleterious-ness), was evaluated using MotifTools (https://github.com/hoondy/MotifTools). D-score was calculated based on the difference between sequence specificities of reference to alternative sequence.

$$\text{D-score} = \text{motif-score}_{\text{ref}} - \text{motif-score}_{\text{alt}} = -10 \cdot \log_{10}\left(\frac{p\text{-value}_{\text{ref}}}{p\text{-value}_{\text{alt}}}\right)$$

We only considered positive D-scores, which denote a variant that decreases the likelihood that a TF will bind the motif (motif-break), and ignored negative D-scores where a variant that increases the likelihood that a TF to bind the motif (motif-gain). For assessing D-score, uniform nucleotide background was assumed, and the p-value threshold of $5e^{-2}$ was used. For each variant that affected multiple RBP binding profiles were ***averaged***(we need to decide if we average or max) over all D-scores.

4.6 Variant Scoring

4.7 Regulatory Network Construction

In order to construct a regulatory network of protein coding genes associated with a given RBP, we first identify which annotation is associated with which protein coding gene. The network we construct is undirected between protein coding genes and consists of a set of genes that a given RBP interacts with. To determine which genes the RBP interacts with, all binding sites of the RBP are intersected with all annotations (4.2). With the additional information of the associated gene given the annotation, we compile a list of all protein coding genes associated with the RBP. A unique list is determined and such a set of genes is determined to be the network of genes associated with that RBP. This is performed across each RBP in order to obtain a set of genes associated with each RBP.

4.8 RNA Binding Protein Prioritization

4.8.1 Logistic regression and regulation potential (add the DEseq analysis, have the software version clearly labeled)

To prioritize the RBPs we use a logistic regression approach. Our goal is to assess the regulatory potential (positive or negative) that the RBPs have on their respective gene associated targets. For each RBP we perform a logistic regression to evaluate the individual regulatory potential on a set of its target genes. Our explanatory variable, y, in the logistic regression consists of a vector of 1s and 0s with vector length equal to the number of protein coding genes, xxx. For each gene, the corresponding position in the vector y is equal to 0 if that gene is not in the regulatory network, and 1 if it is. This vector is rather sparse, containing many more 0s than 1s. The x variable consists of a vector of protein coding gene differential expressions. We determine these differential gene expression values for 24 different cancer types, allowing us to obtain 24 different regulatory potentials, depending on tissue type. Expression data is downloaded from TCGA Data portal. The count data from RNA-Seq is used in the analysis. The goal in differential expression is to allow for the detection of an extreme value for positive or negative coefficient in the logistic regression in order to indicate upregulation or downregulation, respectively. To calculate the differential expression, DESeq2 (R Bioconductor package DESeq2 v3.5) is used, due to its flexibility in allowing varying numbers of tumor and normal samples. All cancer and normal samples are merged into categories of cancer and tumor, respectively, to determine an appropriate differential expression. Therefore, each RBP network for each cancer type satisfies a logistic regression, and the regulatory potential is inferred from the value of the coefficient. The associated p-value is also an indication of the statistical significance that such a regulatory potential exists.

4.8.2 Survival analysis

We also perform a patient wise regulatory potential logistic regression, where the differential expression is determined as the individual expression fold change from a population mean. Each individual for a given cancer type is given a regulatory potential for each RBP, allowing for the regulatory potential of certain RBPs to serve as a prognosis marker. For each patient, the matching clinical XML data files are parsed for survival time. Patients who are alive use the number of days since the last follow-up as a censored measure of survival time. Survival curves are plotted, with 95% confidence intervals.

4.9 Resource and software accessibility

This RNA variant prioritization tool is made available as an open source python source at xxx. The website contains details on usage, examples, resources, and dependencies. A system with 10gb of RAM is recommended to avoid slowed performance for variant sets with sample size less than 1 million. We also provided a genome wide pre-built PASPort score for every basepair on the genome (hg19 version of genome). Users can directly query the annotation

and functional impact score from xxxxxxxx (link). We also released the RBP-gene regulatory network at xxxxxxxx (link).

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