
Supplementary information

**Sequence determinants of human gene
regulatory elements**

In the format provided by the
authors and unedited

Supplementary information for

Sequence determinants of human gene regulatory elements

Biswajyoti Sahu^{1,2}, Tuomo Hartonen¹, Päivi Pihlajamaa¹, Bei Wei^{3,4}, Kashyap Dave³, Fangjie Zhu⁵, Eevi Kaasinen^{1,3}, Katja Lidschreiber^{6,7}, Michael Lidschreiber^{6,7}, Carsten O. Daub^{7,8}, Patrick Cramer^{6,7}, Teemu Kivioja¹ and Jussi Taipale^{1,3,5*}

1. *Applied Tumor Genomics Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland*
 2. *Medicum, Faculty of Medicine, University of Helsinki, Helsinki, Finland.*
 3. *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden*
 4. *Department of Genetics, Stanford University School of Medicine, Stanford, CA, USA*
 5. *Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom*
 6. *Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany*
 7. *Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden*
 8. *Science for Life Laboratory, Stockholm, Sweden*
- * Corresponding author: Jussi Taipale (ajt208@cam.ac.uk)

The supplementary information contains:

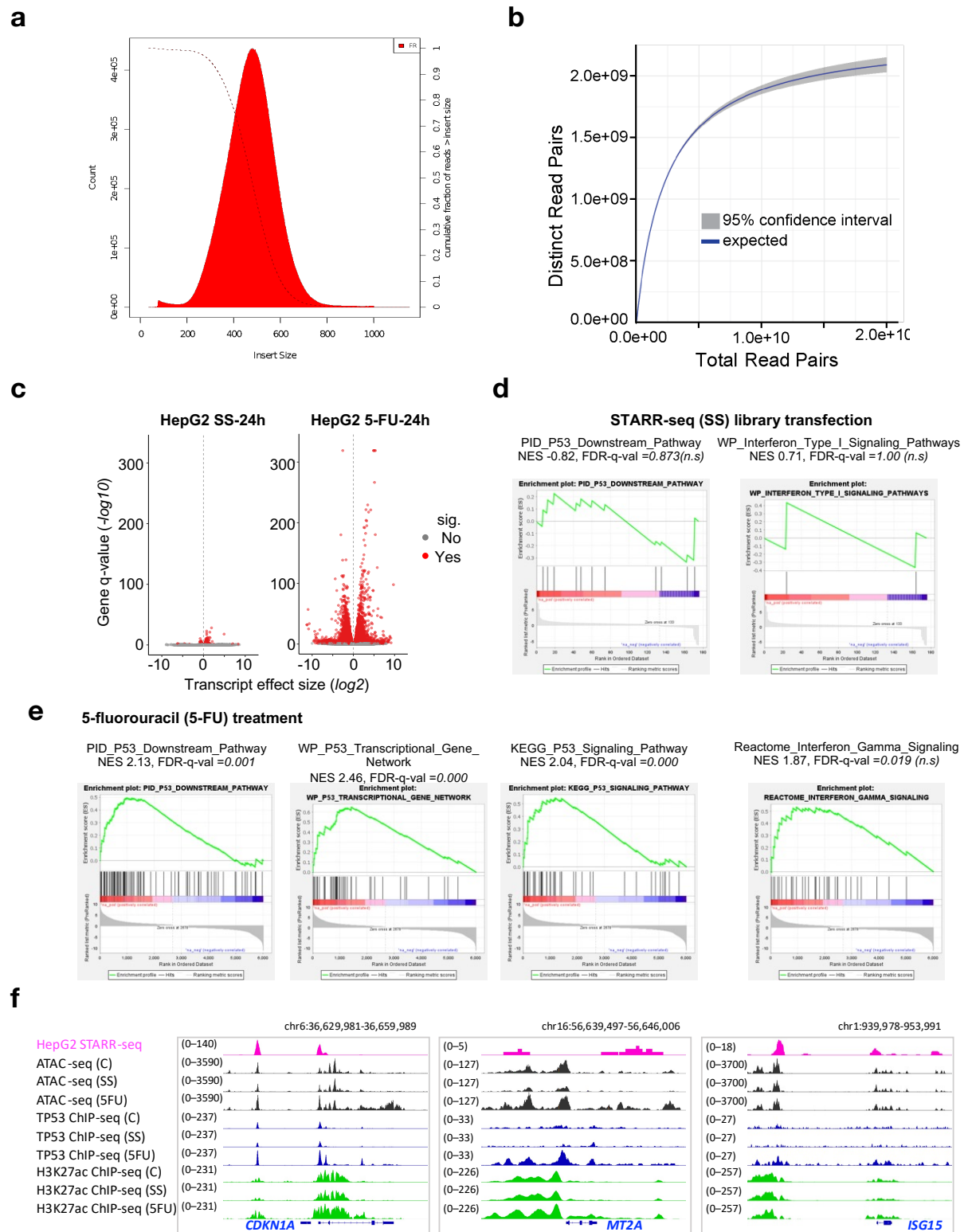
Supplementary Figures 1-4

Supplementary Note

Supplementary Methods

Supplementary References

Supplementary Tables 1-12 (in a separate Excel file)



Supplementary Fig. 1. The complexity of the genomic DNA input STARR-seq library and analysis of cellular alarms in response to reporter library transfection.

a, Histogram showing the insert size distribution of input library comprising of human genomic DNA fragments.

b, Genomic STARR-seq input library complexity estimation. Blue line shows the expected number of distinct read pairs (y-axis) given a (hypothetical) number of sequenced read pairs (x-axis) estimated based on the copy numbers of the sequenced read pairs. The gray band shows

the confidence interval based on bootstrapping. Extrapolation of the sequenced random sample gives an estimate of over 2 billion distinct read pairs (fragments) in the library (see **Methods** for details).

c, Differential gene expression in response to STARR-seq library transfection and p53 induction. RNA-seq was performed from HepG2 cells 24 h after genomic STARR-seq library transfection (SS) or 5-fluorouracil (5-FU) treatment. Fountain plots show differentially expressed genes (q -value < 0.05 ; see **Methods**) from SS- and 5-FU samples compared to mock. The transcript effect size is the estimated effect size of the transcript with the smallest P value. Note that there are only 180 differentially expressed genes induced by the plasmid transfection (*left*), whereas 5-FU treatment results in 6273 differentially expressed gene (*right*).

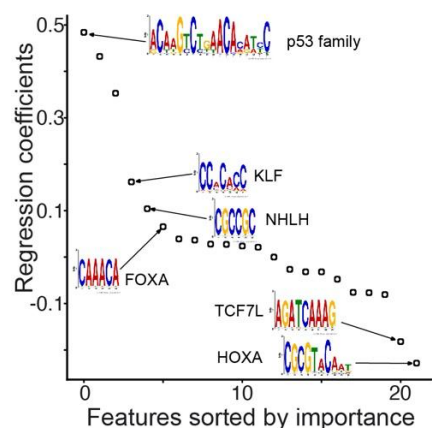
d, e, Gene set enrichment analysis for p53 and interferon signaling pathways from differentially expressed genes in response to STARR-seq library transfection (**d**) and 5-FU treatment (**e**) in HepG2 cells. Note that there were no significantly enriched gene sets in the plasmid transfected cells; the two enrichment plots shown for reference in (**d**) have q -values of 0.873 and 1.00, respectively. However, robust and statistically significant activation of p53 targets as well as interferon gamma signaling pathway was observed in 5-FU-treated cells (**e**). The gene sets used in the analysis were obtained from the molecular signatures database (MSigDB).

f, Genome browser snapshots of three genomic loci, a p53 target gene (*CDKN1A*), and two target genes of the interferon pathway (*MT2A* and *ISG15*) showing enhancer activity from genomic STARR-seq data, chromatin accessibility from ATAC-seq, TP53 binding as well as H3K27ac from ChIP-seq data in HepG2 cells. Traces from cells transfected with STARR-seq plasmid, cells treated with 5-FU as well as control (mock) cells are shown. Note that plasmid transfection itself does not induce changes in TP53 binding to its target genes or changes in chromatin accessibility or H3K27ac at IRF target gene loci. Collectively, the results shown in **c-f** demonstrate that in HepG2 cells, the plasmid transfection does not elicit strong cellular alarm responses (see also ref. ¹).

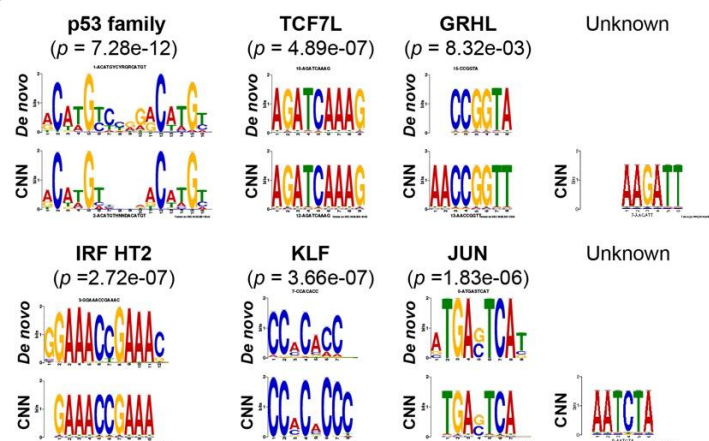
a

<i>De novo</i> motif	TOMTOM	<i>p</i> -value	sites	<i>De novo</i> motif	TOMTOM	<i>p</i> -value	sites
	TP53	7.5e-6068	31351		FOXO3	8.4e-011	139162
	TP53	2.9e-242	1832		TP53	1.2e-010	471
	IRF3	1.8e-110	1049		IRF3	1.4e-008	142
	ELK1	4.9e-072	230761		GRHL	5.8e-007	995
	JDP2	4.6e-056	6212		TCF7L	3.2e-006	860
	TP53	2.1e-046	4018		FOXD3	5.6e-006	49359
	KLF1	6.8e-027	103174		TP73	2.9e-005	55
	ERG	5.2e-020	65121		HNF4	1.3e-004	25014
	IRF4	4.2e-015	168		IRF5	1.9e-004	35794
	NHLH1	1.1e-013	105668		SP8	9.6e-003	474
	HNF1	1.5e-011	882		HOXA13	1.3e-002	3657

b



c

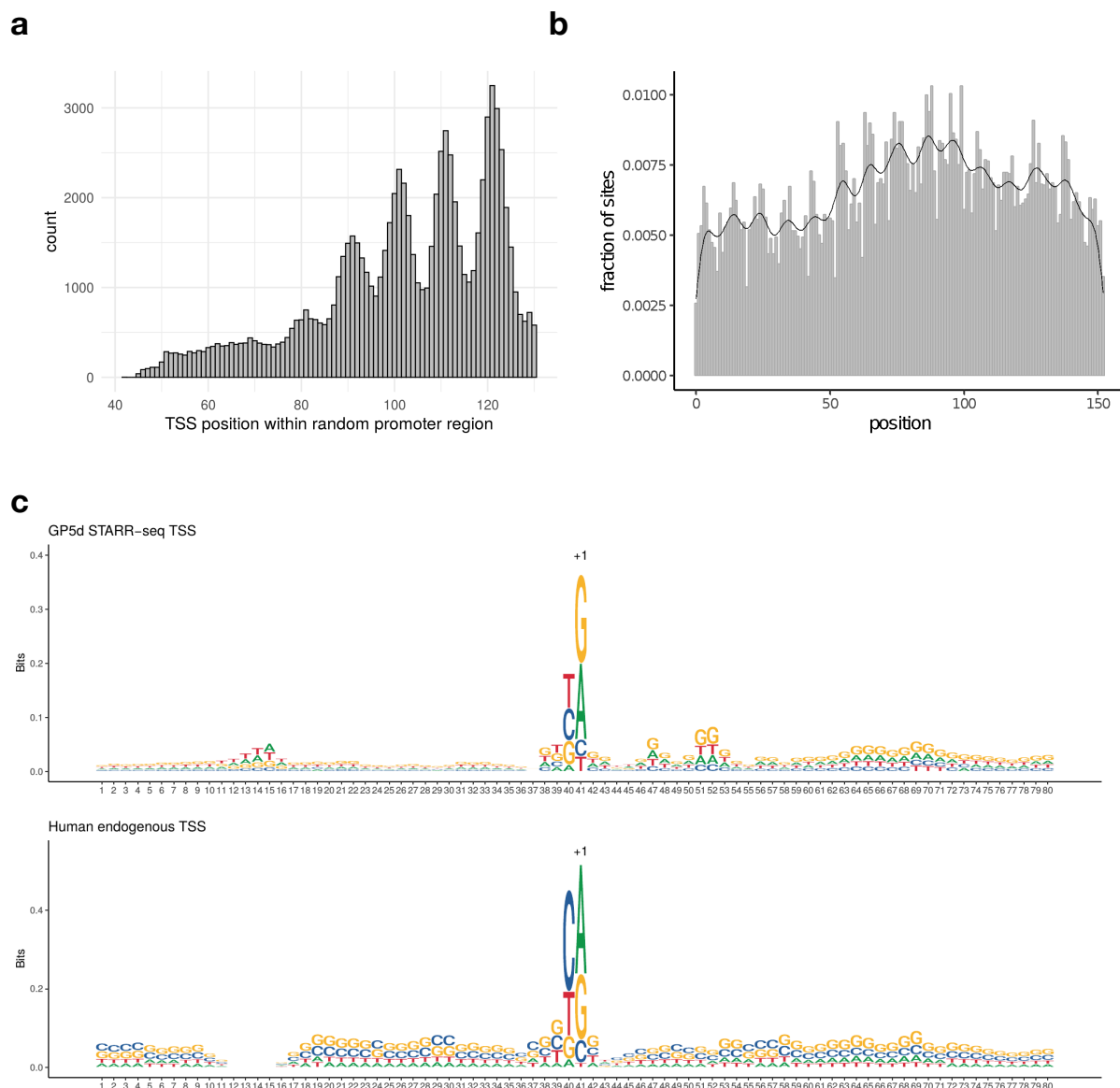


Supplementary Fig. 2. De novo motifs enriched from random enhancer STARR-seq data in GP5d cells and from sequences learned by the CNN.

a, De novo motifs enriched from random enhancer STARR-seq data analyzed using STREME program. The *P* values and the numbers of sequences matching the motif are shown as calculated by the STREME algorithm (no additional multiple hypotheses correction) along with the motif identity from TOMTOM (see **Methods** for details).

b, Regression coefficients (y-axis) for the 22 *de novo* motifs from random enhancer data (from panel **a**). The motif features with strongest regression coefficient values are highlighted. The regression model using the *de novo* motifs obtained a similar area-under precision recall curve (AUprc) score than the simple logistic regression using the 880 HT-SELEX derived motifs (for both, AUprc=0.55).

c, Comparison of the *de novo* motifs discovered from the top 0.5% highest-scoring random sequences according to the GP5d random enhancer CNN model (lower motif of each pair) and the best matching GP5d random enhancer STARR-seq *de novo* motif (top motif of each pair). If no significant match was found from the GP5d random enhancer STARR-seq *de novo* motifs (TOMTOM *P* value threshold 0.05), only the motif learned by the CNN model is shown. Bonferroni-corrected empirical *P* values reported by the TOMTOM program for the comparison between *de novo* motifs discovered from random enhancer STARR-seq and CNN-learned sequences are indicated for each pair shown in the figure.

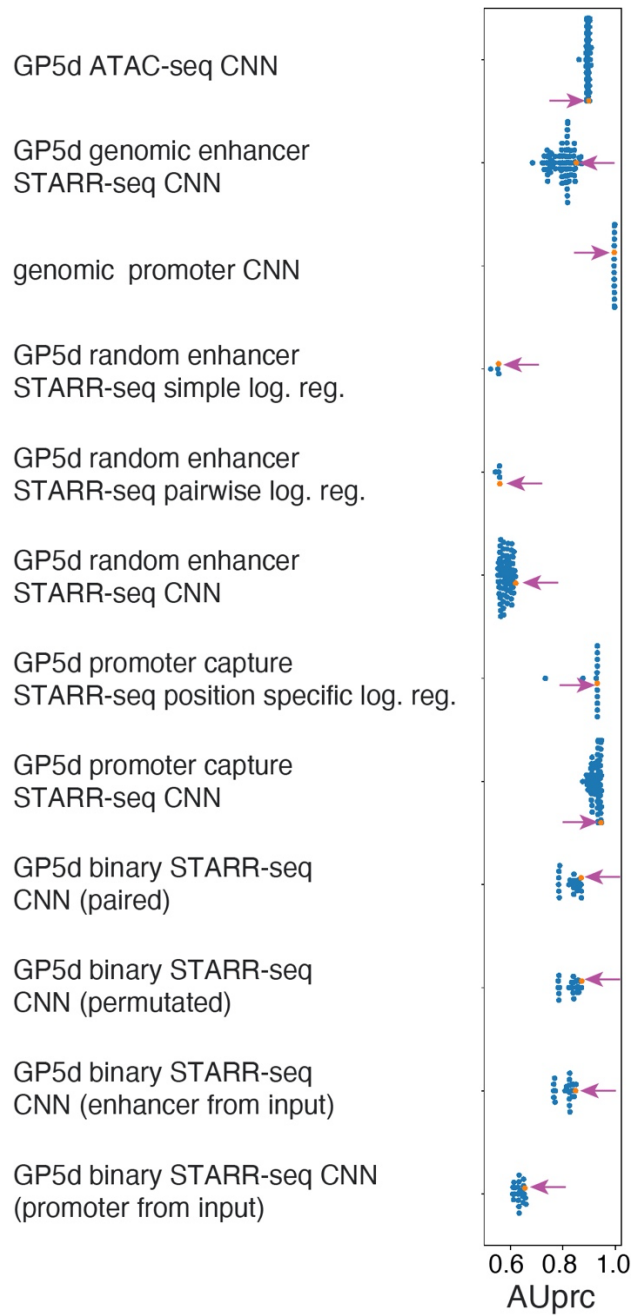


Supplementary Fig. 3. Features discovered from random promoter and random enhancer STARR-seq measurements.

a, Template switch reads used for positioning the TSS show a 10-bp periodicity relative to the STARR-seq vector.

b, Positioning of the p53 motif matches in the random enhancer. The fraction of p53 motif match sites starting at each position is shown. Black line shows the smoothed density estimate.

c, TSS enriched from random sequences (*top*) show similar features as human endogenous TSS (*bottom*). The G-rich element detected downstream of TSS from the random promoter STARR-seq data is also present in the human genome. However, to our knowledge it has not been recognized before, potentially because the feature is obscured by the general GC anisotropy (more guanines on the plus strand) both upstream and downstream of the TSSs which has been reported earlier (see ref. ²) and may partly be caused by mutational mechanisms.



Supplementary Fig. 4. Testing machine-learning models for optimal hyperparameter combinations.

All tested hyperparameter combinations for each machine-learning classifier scored on unseen test data. Classes are balanced in each test set meaning that a classifier assigning samples with random labels with equal probabilities would obtain an AUprc score of 0.5. The final model for each hyperparameter combination was selected by maximizing binary accuracy on a separate validation dataset, unless otherwise stated in the **Methods**. Each dot in the swarm plot represents the test set AUprc for a final model for a certain set of hyperparameters. The exact hyperparameter combinations tested are listed in **Supplementary Table 8**. The model names on y-axis correspond to the given model names in **Supplementary Tables 8, 9**. The best model for each task (marked with an arrow) was selected based on validation data across all tested hyperparameter combinations for that task. The exact hyperparameters of the chosen models are listed in **Supplementary Table 9**.

Supplementary Note

Supplementary text to support the main conclusions.

1) Few TFs display strong transcriptional activity in cells

The main evidence for this finding is presented in **Fig. 1**, which provides details of the massively parallel reporter assay (MPRA) designs, experiments, and data analyses used in this study (**Fig. 1a, b**) and utilizes the results from STARR-seq experiments using the TF motif library (motif-STARR-seq; **Fig. 1c, d**) to analyze enhancer activity in human cells. In the STARR-seq experiments, the active enhancer elements can be identified by transfecting cells with a plasmid library in which the DNA fragments of interest are cloned downstream of a reporter gene, and by analyzing the reporter-specific RNA to identify self-transcribed active elements (**Fig. 1b**).

The rationale for designing the TF motif library for STARR-seq experiments was to systematically test the enhancer activity of known TF motifs in isolation (one copy) and in multiple copies (**Fig. 1a, i**). Thus, the motifs were placed in two motif-depleted sequence contexts in different orientations and spacings. The motifs used in the design were collected from previous HT-SELEX studies as described in **Supplementary Methods** (section **Motif collection**). HT-SELEX is an *in vitro* assay for determining the DNA binding specificities of individual TFs through systematic evolution of ligands by exponential enrichment, and a total of 1121 HT-SELEX-derived motifs were included in the TF motif library design in this study. In addition, a degenerate base N was added at every position of each motif one position at a time to generate in total 30,123 mutant consensus sequences. The motif library comprises a total of 92,918 individual sequence patterns including these 1121 TF consensus sequences and 30,123 mutant consensus sequences in two motif-depleted sequence contexts (to control for effect of flanking bases). Each pattern only contained one type of consensus sequence, present once, twice and/or three times. Multiple consensus sequences were arranged in different spacings and orientations relative to each other. In the figures, dimeric motifs are indicated by orientation with respect to core consensus sequence (GGAA for ETS, ACAA for SOX, AACCGG for GRHL and GAAA for IRF; HH head-to-head, HT head-to-tail, TT tail-to-tail, followed by gap length between the core sequences). **Supplementary Table 5** describes the naming of the motifs in each figure.

The main advantage of this approach is that it enables measuring of the functional enhancer activity of distinct *in vitro*-derived TF motifs *in vivo* in human cells. Based on the MPRA experiments, we identified the most active motifs in GP5d cells (**Fig. 1c**). The most active motifs displayed comparable activities when placed in different sequence contexts (**Extended Data Fig. 1a**), and their relative activities were consistent across experiments using two different CpG-free basal promoters, δ 1-crystallin and EF1 α promoters (**Extended Data Fig. 1b**). The mutated TF motif patterns enabled generation of activity-based position weight matrices (PWM) for the TFs (**Fig. 1d** and **Extended Data Fig. 1d**). So far, the PWMs have been generated either on the basis of the *in vitro* TF-binding specificities from the SELEX experiments or as enrichment of sequence features within ChIP-seq peaks for a particular TF. Compared to these previous approaches, our results provide a direct measurement of enhancer activity of each consensus motif and its mutated variations in human cells.

The main limitation of the motif-STARR-seq experiment stems from the facts that many TFs bind to highly similar motifs and the binding specificities for all individual TFs are not yet known. However, although the assay measures activities of consensus sequences and/or motifs, the TFs or groups of TFs that bind to the sequences can in most cases be inferred from the specific motifs and TF expression levels³. We used RNA-seq data to determine the expression levels of the TF families with strongest detected TF motif activity, as shown in

Extended Data Fig. 1c. Such combined analysis revealed that TFs that are expressed at a relatively high level in GP5d cells can bind to the strongly active motifs (average 102 transcripts per million (tpm) vs. 26 tpm for all genes and 20 tpm for all TFs from ref. ⁴); however, the correlation between motif activity and expression of corresponding TFs was weak (**Fig. 1c, Extended Data Fig. 1a-c**). This is likely caused by the facts that: 1) different TFs have different binding energies, 2) different TFs have different transcriptional activator activities, 3) mRNA expression level does not fully determine protein expression level, and 4) mapping of motifs to TFs is not perfect. Since all expressed TFs that are capable of transactivating through similar sequences can contribute to the measured enhancer activity, assigning the measured enhancer activity to a specific TF is not always possible. To acknowledge this limitation in our study, in the figures the motifs have been named according to the TF class or subclass for the cases where HT-SELEX motifs for several TFs are highly similar. Same principle has been applied also when specificities of closely related TFs have not been measured yet but can reasonably be expected to be similar based on the sequence homology.

Another potential limitation of the MPRA approach could be a strong activation of cellular alarm responses due to experimental conditions, as described previously (see ref. ¹). Based on the motif-STARR-seq data, only three other motifs, representing interferon regulatory factor (IRF), grainy-head like (GRHL) and E26 transformation-specific (ETS) TFs had activity that was within 1% of the maximal activity observed for the p53 family motif (**Fig. 1c**, dotted line). Since the two most enriched motifs, p53 and IRF, are bound by TFs that respond to cellular alarm signals such as DNA damage (p53) and cytoplasmic DNA (IRF), we studied the extent of these alarm signals in our experimental system by performing control experiments in which the HepG2 cells were transfected with the genomic STARR-seq library or treated with 5-fluorouracil (5-FU) to induce p53. Cellular responses were analyzed by RNA-seq for gene expression, ATAC-seq for chromatin accessibility, and ChIP-seq for p53 binding as well as for active histone mark H3K27ac. Importantly, we found that plasmid library transfection itself caused very minor changes in the cellular transcriptome (**Supplementary Fig. 1c**) or p53 binding, chromatin accessibility and H3K27 acetylation at p53 and IRF target genes (**Supplementary Fig. 1f**). Gene set enrichment analysis showed that p53 target genes were strongly enriched among differentially expressed genes in 5-FU-treated cells, but not in cells transfected with the STARR-seq plasmid (**Supplementary Fig. 1d,e**). These results indicate that in HepG2 cells, the alarm response is mild.

Of note, in all the STARR-seq experiments in this manuscript, the cells were in an unstimulated state. Thus, the activity of some TFs that are active only upon stimulation (for example, hormone- and growth-factor dependent TFs, immune response-related TFs), is not detected under the experimental conditions used.

2) *De novo* enhancers display weak TF spacing and orientation preferences

Fig. 2 utilizes the results from STARR-seq experiments with TF motif library (**Fig. 2a,b**) and random enhancer library (**Fig. 2c-f**) to analyze enhancer activity in human cells. In addition, active TF identification (ATI) assay for measuring DNA binding activity of the TFs (**Fig. 2a**) in the same cell line in which the motif-STARR-seq was performed.

The rationale for comparing the motif-STARR-seq results to ATI results was to study how the enhancer activities of TF motifs correlate to the DNA binding activities of the respective TFs. ATI assay utilizes a library of random DNA sequences that are incubated with nuclear extract from the cell type of interest, and the oligonucleotides that are bound by the TFs are enriched using electrophoretic mobility shift assay³. Comparison of TF motif activity from motif-STARR-seq to TF binding activity from ATI assay revealed only weak correlation between the transcriptional activity and the biochemical activity ($R=0.032$; **Fig. 2a**), indicating

that the TF motifs responsible for the activities are largely distinct. Note also that some TFs can bind to two or more slightly different motifs that can have somewhat different activities (e.g. p53-family motif is bound by all p53 family members, p53, p63 and p73, whereas the p53-specific motif is only bound by p53. Although there are limitations in comparing the results from two different assays, such as different technical properties and sensitivity of each assay, as well as quality of the individual data sets, the weak correlation we observed also reflects the different aspects of TF function measured by these two assays; ATI assay enriches the DNA sequences that are most strongly bound by the TFs expressed in a particular cell type, whereas STARR-seq measures the outcome of enhancer activity as accumulation of specific transcripts in response to TF binding. Our results demonstrate that the TFs with strongest DNA binding activity in a cell are largely different from the strongest transcriptional activators. Moreover, the two strongest binders identified by the ATI assay in GP5d cells (NFI and NRF1) were also reported as strong binders across many human tissues in a previous ATI study³, suggesting that strong binding does not necessarily lead to strong cell type-specific transcriptional activity.

Since the synthetic motif library contains sequences with different copy numbers for each motif, we were able to measure the effect of motif copy number on enhancer activity. For the majority of the TFs, we observed an additive effect as a function of the number of consensus sequences (**Extended Data Fig. 1e**, red horizontal lines), whereas for the strong activators, multiple copies of the motifs result in saturation (**Fig. 2b**). For each motif, fold-change ($\log_2$) compared to input is shown for one versus two sites. Of note, additive effect in the logarithmic space equals multiplication of fold-changes.

In addition to the TF motif library (**Fig. 1a**, i), we used a library comprising synthetic random 170 bp sequences (**Fig. 1a**, iii) cloned downstream of the reporter gene in the STARR-seq experiments. These two approaches complement each other: while TF motif library enables studying the enhancer activity of different variations of predefined sequence patterns, the experiment using the random library is free from any *a priori* assumptions of what constitutes an active enhancer in human cells. Active motifs identified in the experiments using these two different library types were highly similar (**Fig. 1c**; **Extended Data Fig. 2b**), illustrating the robustness of the assay. Furthermore, comparison of replicate experiments for random enhancer libraries revealed high reproducibility across different $\log_2$ fold-change values (**Extended Data Fig. 2a**), demonstrating the high dynamic range of the assay. Note that while p53 and IRF are the strongest activators, also colon cancer-specific TF activity can be reliably measured from the random enhancer library. These results show that regulatory elements can readily be generated in an experiment by selecting sequences from a very high complexity library, although not all such elements would be optimized or as highly active as the most active elements in the genome. Furthermore, the active motifs enriched from the random enhancer library were also largely similar between the two different cell types, GP5d and HepG2 (**Fig. 2f**), and only few TFs were found to be specific for each cell type. These include for example GRHL in GP5d, TEAD and ATF4:CEBPB in HepG2, and TFs that correspond to the known deficiency of the HepG2 cells in interferon signaling (IRF3)⁵.

The random enhancer library also enabled studying the effect of motif spacings within the active sequences. In general, the spacing and orientation preferences between TFs were weak, but we observed few specific motif pairs with significantly overrepresented spacing preferences (**Fig. 2c**). The sequence logos shown in **Fig. 2c** are the most enriched spacing and orientation for each pair according to *P* value; fold-change is its count compared to average of all others, and the adjusted *P* value is calculated by comparing it to all others (one-sided Fisher's exact test) and correcting for the total number of orientations and spacings tested for the pair (see **Supplementary Methods**; **Supplementary Table 5**). Since the number of detected motif pairs with spacing preferences was small, we wanted to estimate whether the statistical power of our assay was sufficient for detecting specific spacings and orientations. We conducted a

power analysis that simulates different effect sizes and motif match thresholds with the same data size as was used in the motif spacing analysis shown in **Fig. 2c**. The analysis showed that the assay has sufficient power to detect moderate effects (fold-change ≥ 3 if 1% of the pairs had a specific interaction) with high probability (> 0.77) using the same motif match threshold as in the figure (10^{-4}) which is consistent with the interactions shown. Moreover, even with a more stringent threshold (6×10^{-5}) that corresponds approximately to a motif with information content of 15 bits typical for TFs, the assay should be able to detect fold-changes ≥ 5 (probability > 0.92), but only p53/p63 motif interactions were detected with this threshold. Thus, although it is difficult to rule out that some specific interactions could have been missed, the power analysis indicates that if strong specific interactions existed between individually enriched motifs in the assay, we would have detected them with very high likelihood.

In addition, we analyzed the motif spacing from mean of mutual information between 3-mer distributions plotted as a function of distance separating the 3-mers in GP5d random enhancers (**Extended Data Fig. 2e**) and binary STARR-seq enhancers (**Extended Data Fig. 2f**) from the library in which both the promoters and the enhancers comprise synthetic random sequences (**Fig. 1a**, iv). We observed that pairwise dependency between 3-mer distributions measured with mutual information varies with a period of approximately 10 bp and decays as a function of distance separating the 3-mers, indicating that most of the dependencies in the enhancers are short-ranged (< 50 bp).

In addition to motif matching, we analyzed the random enhancer STARR-seq sequences using different machine-learning models in order to understand what features are needed to separate sequences acting as active enhancers from inactive sequences (**Fig. 2d, e**). Data from the random STARR-seq experiments is well suited for machine-learning analyses for three main reasons: 1) In the STARR-seq experiment, the transcriptional machinery of the cell acts as a filter that lets through sequences that can drive transcription. Thus, the experiment can be naturally modeled as binary classification where we aim at finding the features that separate the active sequences from the inactive ones. 2) Background model of the random STARR-seq experiments is much simpler than that of the genome; for example, synthetic random sequences lack the background from mutational processes not directly related to transcription. 3) Number of distinct training samples available is high, *e.g.* ~ 9.2 million sequences for the GP5d random enhancer STARR-seq (see **Supplementary Table 7**).

We started with simple logistic regression that uses a set of 880 previously published TF binding motifs as features (**Fig. 2d**). This model is capable of explaining the activities of the STARR-seq enhancers with a linear combination of effects of known individual TF binding motifs (see section **Logistic regression classification** in **Supplementary Methods** for details). This model performed similarly in classification to a model that was trained using the 22 *de novo* motifs discovered from the random enhancer STARR-seq data (see **Supplementary Fig. 2a,b**), indicating that the L1-regularization and the used training scheme were capable of finding the correct active features. We then created a pairwise logistic regression model (see **Supplementary Methods**), that uses in addition to the 880 individual TF motifs terms that account for cooperative pairwise binding of each TF with itself and the top 20 most important TFs (according to the regression coefficient absolute values from the simple logistic regression) with all other TFs from the set of 880 binding motifs (in total 19,150 features). All possible pairings could not be tested due to computer memory requirements, but we reasoned that it would be very unlikely for individually inactive motifs to have very important cooperative interactions. Nevertheless, we also trained convolutional neural network (CNN) models similar to recent successful approaches, used for example in refs ^{6,7}.

One strength of the CNN approach is that it learns the important predictive features from the data allowing, in principle, learning any kind of sequence features. In our design of the random enhancer STARR-seq CNN, the first layer filter size was optimized between 5 and

9 (see **Supplementary Table 7** and section **Convolutional neural network classification in Supplementary Methods** for details), and the number of filters in the first layer of the CNN models was capped at 256, which with the minimum dropout of 0.3 allows the models to learn approximately 180 different 5 or 9 bp long filters representing binding motifs. The subsequent dilated convolutional layers (up to 7 layers in total) can then learn to combine these filters into longer motif representations and interactions between them. The limited number of strongly active TF binding motifs detected using motif matching (**Fig. 2b**), *de novo* motif mining (**Extended Data Fig. 2d**; **Supplementary Fig. 2a**) or logistic regression (**Fig. 2d**) analyses strongly suggest that this limitation of the CNN model size is reasonable. The CNN classifier performed better in classifying the GP5d random enhancer data than the logistic regression or gapped k-mer SVM models (see section **Note on optimal classification of random enhancer STARR-seq data in Supplementary Methods** for more discussion).

A clear benefit of the logistic regression approach is that the importance of each designed feature is straightforward to read from the regression coefficient values (**Fig. 2d**). With CNN models, interpreting the features learned by the model and their importance towards the classification is not as straightforward, although considerable progress has recently been achieved (e.g. refs ^{6,7,8}). We used a barrage of different interpretation strategies to inspect the features learned by the random enhancer CNN model (**Fig. 2e**; **Supplementary Fig. 2c**; **Extended Data Fig. 3c**; **Extended Data Fig. 4**). First, we observed that if we create “artificial enhancers” by embedding one or two instances of known binding motifs into inactive input library sequences (**Fig. 2e**), the enhancer probabilities predicted by the random enhancer STARR-seq CNN agree well with the results from the motif matching (**Fig. 2b**) and logistic regression (**Fig. 2d**) analyses. This shows that the CNN has learned similar effects for the known TF binding motifs than the logistic regression model. The core sequences of the motif features used by the logistic regression model were typically also learned by the CNN, but the weaker flanking bases of the motifs were more variable (**Extended Data Fig. 3c**). Other types of sequence features, or interactions between features (**Fig. 2e**) might also exist that are important in classification but not captured by the logistic regression model (see **Supplementary Methods**). Moreover, *de novo* motifs from unseen *in silico*-generated random sequences classified as active enhancers by the CNN model are highly similar to *de novo* motifs from the random enhancer STARR-seq (**Supplementary Fig. 2c**). Of note, CNN also identifies similar features than the enrichment analysis shown in **Extended Data Fig. 2d**.

Re-creating the important binding motifs from the logistic regression model using the per-base importance learned by the CNN (**Extended Data Fig. 3c**, see section **Interpretation of convolutional neural network classifiers in Supplementary Methods**) revealed that most of the motifs learned by the CNN were different from the HT-SELEX derived motifs at the less defined positions (e.g. ELF5, SP1), for some motifs, only part of the HT-SELEX motif was used by the CNN (e.g. TCF7, TP63), and some motifs seemed not to have been learned at all (e.g. NFAT5, SMAD3). TF-MoDISco⁹ analysis further supported this by recovering only part of the motifs used by the logistic regression classifier (**Extended Data Fig. 4**). The machine-learning classifiers are regularized and trained so that they aim at finding simple features that generalize well to new datasets. The logistic regression models were regularized using L1 norm and the CNN models with dropout layers. Thus, it is not surprising that the less information-rich parts of TF binding motifs are not necessarily deemed important for the classification task. This does not necessarily mean that they are not important for biology and care must be taken when interpreting the features used by machine-learning models, since the partial binding motif might be as good for classification as the full-length motif according to the used validation data, so it is not necessary to learn the more complex full-length motif.

3) Methylation status affects enhancer activity both positively and negatively

Analysis of the role of CpG methylation on enhancer activity is shown in **Fig. 3**. In addition, **Fig. 3** shows the RNA-sequencing data that was used for analyzing cell type-specific expression of TFs (**Fig. 3a**). Genomic STARR-seq was used for studying the effect of CpG methylation on enhancer activity in GP5d cells (**Fig. 3b-d**). Furthermore, previously published bisulfite sequencing data from GP5d cells (from ref. ¹⁰) was used for detecting genome-wide CpG methylation at the endogenous genomic loci (**Fig. 3b**) and ATAC-seq was used for mapping the accessible chromatin regions in the genome so that open and closed chromatin regions could be correlated with genomic STARR-seq activity in GP5d and HepG2 cells (**Fig. 3b, e**). Genomic STARR-seq experiments were also performed in *TP53*-null GP5d cells (**Fig. 3b**).

To enable analysis of the effect of DNA methylation on transcriptional activity, we developed a MPRA vector that is devoid of CG dinucleotides: in designs i to iii, the Lucia reporter gene is driven by CG depleted minimal $\delta 1$ -crystallin and EF1 α promoters, whereas in design iv, the promoter is replaced by 150 bp random DNA sequences (see **Fig. 1a, b**; **Methods** and **Supplementary Tables 1, 2**). Since the vector backbone is devoid of any CG nucleotides, *in vitro* methylation of the plasmid library only methylates the DNA fragments of interest. The enhancer activity of methylated and unmethylated genomic libraries can then be analyzed in parallel transfections. In the genomic STARR-seq experiments using methylated and unmethylated libraries, we did not observe a very strong overall effect from DNA methylation on the landscape of enhancer activity (**Fig. 3b**). However, the activities of individual enhancers were affected in both directions; we also found differential enrichment for specific TF motifs containing CG dinucleotides that were consistent with the reported binding specificities for methyl-plus and methyl-minus TFs (**Fig. 3d**; see ref. ¹⁰). These results demonstrate that differences in TF activity in the presence and absence of DNA methylation can be detected using a genomic STARR-seq assay.

4) Genomic analysis reveals three types of transcriptionally active enhancers

Genomic enhancer activities were compared with chromatin features in **Fig. 4**, which shows the results from STARR-seq experiments with genomic DNA library in two cell lines, GP5d and HepG2, as well as analysis of chromatin accessibility using ATAC-seq and TF binding and epigenetic features using ChIP-seq in the same cell lines (**Fig. 4a, b**). This analysis revealed six types of gene regulatory elements (**Fig. 4c**).

The rationale for using the genomic DNA library was to study the enhancer activity of sequence features in their native genomic sequence context. In contrast to rationally designed and randomly synthesized DNA fragments used in the STARR-seq experiments in **Fig. 1** and **Fig. 2**, the genomic DNA library comprises ~500 bp fragments of the human genome cloned downstream of the reporter gene (**Fig. 1, ii**). The active enhancers were identified by calling the peaks from the STARR-seq-enriched RNA fragments against the plasmid input sample using MACS2¹¹ (see **Methods**). The high complexity of the library (2.09×10^9 distinct clones; **Supplementary Fig. 1a,b**), replicate concordance and excellent signal-to-noise ratio allowed detection of enhancer activity at ~1.5 bp resolution (**Extended Data Fig. 5a, b**; see **Methods**). The genome browser snapshot in **Extended Data Fig. 5b** shows a near base-pair resolution at the STARR-seq peaks, indicating that active fragments drive gene expression and are converted to cDNA, recovered and sequenced at a high probability, speaking against any potential bottleneck-effects or undersampling. Also note that cell type-specific STARR-seq signals agree with tissue-specific TF binding (highlighted with pink boxes in **Extended Data Fig. 5b**): TP53 binds at intronic enhancer in both GP5d and HepG2 cells, but stronger STARR-seq signal in HepG2 cells is observed at the enhancer upstream of the *BBC3* gene that co-localizes with specific HNF4A binding in HepG2. Since the active enhancers identified by the genomic

STARR-seq experiments can be mapped to their original loci in the human genome, the data can be compared with other functional genomic data sets. Here, we have used ATAC-seq data sets to identify open, transposase-accessible regions in the chromatin, and ChIP-seq to map epigenetic features associated with enhancer activity (such as H3K27ac) and repressed chromatin (such as H3K9me3 and H3K27me3). In addition, ChIP-seq data for CTCF is used for identifying structural chromatin elements such as boundaries of regulatory units. Of note, there is much higher overlap between the transcriptional activator TFs p53 and FOXA1 ChIP-seq and STARR-seq than between the architectural chromatin protein CTCF and STARR-seq (**Extended Data Fig. 5c**).

The strength of the approach utilized in **Fig. 4** is in combining different, complementary data sets to classify human gene regulatory elements. Traditionally, open chromatin sites have been considered to contain active gene regulatory elements, and ATAC-seq can identify both active promoters and enhancers. However, chromatin openness is an indirect measure for activity, and the functional consequence of the elements identified using ATAC-seq needs to be validated using other methods. Genomic STARR-seq, on the other hand, measures the functional enhancer activity of genomic sequences in an unbiased manner in an episomal system. We performed predictive modeling using Lasso regression to test whether these datasets can predict differences in gene expression. This analysis clearly shows that ATAC-seq and STARR-seq both independently predict differential gene expression, and thus we utilized these data sets to classify human gene regulatory elements. This analysis revealed three different types of active enhancers: closed chromatin enhancers (STARR-seq only), classical enhancers (both signals) and chromatin-dependent enhancers (ATAC-seq only).

We define six classes of gene regulatory elements based on the integrative analysis of different functional genomics data sets (**Fig. 4a-c**). There are previous reports that have classified enhancers for example as “active”, “primed”, and “poised” (see, e.g. ref. ¹²). This classification did not consider the functional enhancer activity measured by a reporter assay, which gives an additional layer of information that is used for classification in this manuscript (**Fig. 4c**). However, the different classifications are partly overlapping. Here, we define cryptic enhancers as STARR-seq+, ATAC-seq- sites that show enrichment for H3K9me3 and/or H3K27me3 repressive chromatin marks. Thus, these resemble previously reported class of “poised” enhancers that are characterized by H3K27me3¹². As shown in **Fig. 4a, c**, both classical and chromatin-dependent enhancers are characterized by a strong H3K27ac signal. At closed chromatin enhancers the H3K27ac mark is weaker, whereas TF binding is clearly observed to these genomic loci and enhancer activity is detected using STARR-seq (for example STARR-seq signal overlapping with p53 binding at the intron of the *RRM2B* gene in both GP5d and HepG2 cells; **Fig. 4a**). STARR-seq peaks in closed chromatin represent two element classes: closed chromatin enhancers (no repressive histone marks) and cryptic enhancers (overlap with repressive histone marks such as H3K9me3; see **Fig. 4a**). Overlap analysis revealed that cryptic enhancers do not show overlap with the H3K4me1 mark that is considered to mark the “primed” enhancers, but around one fourth of the closed chromatin enhancers are enriched for H3K4me1 (**Extended Data Fig. 5d**). However, since primed enhancers are considered to be located in open chromatin (see ref. ¹²), closed enhancers do not fully represent the features of “primed” enhancers, but rather represent a distinct category of enhancers.

STARR-seq experiments are limited by the complexity of the input library. In our case, the genomic library complexity is extremely high with estimated 2.09×10^9 distinct fragments covering the human genome (3.2×10^9 bp) with 1.53 bp resolution on average. This provides unprecedented power to detect active enhancer elements in the human genome. A potential risk associated with high library complexity would be undersampling of the input library. However, we designed the experiments carefully to avoid any bottleneck-effects and did not observe any

evidence of undersampling. This is based on analysis of peaks in the genomic library, which would have low resolution if many clones were lost during the experiment and only some would be recovered despite all elements being active. By contrast, we see near base-pair resolution at the peaks, indicating that active fragments drive gene expression and are converted to cDNA, recovered, and sequenced at a high probability (see genome browser snapshots in **Fig. 4a, b**; **Extended Data Fig. 5b**).

As shown in **Fig 2f**, p53 family motif is the strongest activator in both GP5d and HepG2 cells, having the largest fold-change of motif match count over input in each cell line. Similarly, IRF motifs also show strong activity in GP5d cells. We used the genomic STARR-seq data to determine what fraction of all detected elements are regulated by p53 by performing overlap analysis between genomic STARR-seq peaks and the ChIP-seq peaks for TP53. This analysis revealed that p53 is not responsible for most of the enhancer activity detected using STARR-seq. Specifically, 16% and 4.9% of the genomic STARR-seq peaks overlap with a p53 ChIP-seq peak in GP5d and HepG2 cells, respectively (**Extended Data Fig. 5c,d**). Also, only a small fraction of the STARR-seq peaks harbor a p53 family or IRF3 motif (**Extended Data Fig. 5c,d**). These results demonstrate that although p53 family and IRF were the most enriched motifs, the large majority of active enhancers did not contain these elements despite these motifs being the strongest activator in the cells, suggesting that the gene regulatory system of the cell is not dominated by these cellular alarms.

5) Cell type-specific gene expression is driven by few lineage-specific transcription factors; there are promoter-specific sequence features, but no motif enriches specifically at enhancers

Fig. 5 utilizes a binary STARR-seq design in which both promoter and enhancer elements comprise of random synthetic sequences. This design enables measuring the sequence features that contribute to enhancer and promoter activities separately, and also detection of promoter-enhancer interactions. In **Fig. 5a**, motifs enriched at random promoters in three different cell lines were analyzed, and in **Fig. 5b, c** we studied the similarities and differences between motifs enriched at active promoters and enhancers.

The rationale for using DNA libraries comprising synthetic random sequences is to study the determinants of active regulatory elements in an unbiased manner, independently of their sequence context. In the experiments, random sequences that contain sequence elements that can contribute to promoter or enhancer activity in a given cell are enriched without being limited by pre-designed parameters. Ultra-complex random libraries also enable studying far larger sequence space than the entire human genome. While the human genome is too short to encode all possible sequence combinations for TF binding elements, the random library can accommodate a substantially larger number of sequence combinations with potential regulatory activity.

The size of the ultra-complex DNA library prohibits precise determination of initial input frequencies of individual sequences. Thus, our analysis relies on finding common features of different selected sequences instead of their counts. In fact, analyzing enriched features of distinct otherwise random sequences is a very powerful way to distinguish the selected features from the rest of the sequence; it also prevents high copy-number or repeat sequences from biasing motif-mining or motif-spacing analyses. For example, to find enriched TF motifs we counted the motif matches separately in input DNA library and STARR-seq RNA library taking into account the mononucleotide frequencies in each and thus for example differing GC-contents did not affect the enrichment analyses (see **Methods** for details). This approach is not limited to or biased towards assumed motifs, also allowing *de novo* motif analysis or any other sequence feature enrichment analysis.

One of the limitations of many MPRA approaches is that they are less sensitive for detecting repressive effects than enhancing effects due to the minimal promoter typically used in the constructs. We could, however, detect some repressive motifs such as OVOL and CUX (**Fig. 5b**). These results demonstrate that also repressive features can be analyzed using our approach, although with lower sensitivity.

6) TFs can have enhancing, promoting and/or TSS-defining activities

Fig. 6 shows the data generated from a binary STARR-seq experiment in which both promoter and enhancer elements consist of random synthetic sequences. Here, the focus is on the detailed analysis of the positional specificity of sequence motifs at active promoters. Template switch strategy was used for determining the exact position of the TSS within the random promoter sequence (**Fig. 6a**). This enables studying the enrichment and precise position of the sequence features upstream and downstream of the TSS. Cartoon in **Fig. 6a** shows the design of the template-switch chemistry used to capture the 5' sequence of the transcribed RNA using a template switch oligo (TSO) to determine the precise location of the transcription start site (TSS) within the random DNA sequences cloned at the place of the promoter. The sequencing libraries are generated by reverse-transcribing the reporter-specific RNA to cDNA using a reporter-specific primer (orange) and a TSO containing a unique molecular identifier (UMI; brown) and a 5' sequencing adapter (turquoise/green), and by PCR-amplifying the cDNA utilizing primers that bind to the adapters to introduce the Illumina linkers (red) to the final library (3' adapter sequence marked with dark blue is present in the reporter construct).

One of the strengths of our approach in analyzing random sequences is that the enrichment of sequence features can be analyzed independently of their sequence context. Analysis of the sequence features within the random promoters revealed positional preferences for several features, including AT-rich sequence (TATA box) upstream of the TSS and a novel G-rich sequence downstream of the TSS; these elements showed mutually exclusive interaction with the TSS (**Fig. 6b, c**). Many TF motifs preferentially enriched close to the TSS; on average, $\log_2$ fold-change vs. input values for the 50 most enriched TF motifs were 53% higher in the region from -51 to TSS compared to region -100 to -50 (**Fig. 6d**). After identifying the novel G-rich sequence from the random promoters, we then could also detect it from endogenous genomic sequences downstream of TSS (**Supplementary Fig. 3c**); to our knowledge it has not been recognized before, potentially because the feature might be obscured by the general GC anisotropy (more guanines on the plus strand) both upstream and downstream of the TSSs². We also observed that the reads obtained from the template switch analysis preferentially mapped to positions that displayed a 10 bp periodicity relative to the STARR-seq vector (**Supplementary Fig. 3a**). The pattern was consistent with the loading of the RNA polymerase II pre-initiation complex onto the random sequence, as the periodic pattern weakened when the ~100 bp region occupied by the pol II complex included plasmid-derived constant sequences. Similar, but very weak periodicity was also observed in p53 motif positioning at the enhancer, suggesting that plasmid supercoiling, or some sequence feature in the vector makes one side of the DNA more accessible (**Supplementary Fig. 3b**).

We used a convolutional neural network classifier (CNN) trained on the random synthetic STARR-seq promoters to score known variants at the *TERT* promoter^{13,14,15,16} to assess how well a model trained on synthetic STARR-seq sequences has learned to predict the effects of mutations in human regulatory sequences. As shown in **Fig. 6f**, CNN predictor correctly identifies cancer-associated mutations in the *TERT* promoter¹⁶. Of note, the predictor identifies the ETS motifs that are generated by the driver mutations and the predicted promoter probabilities (P_{promoter}) are higher for the mutant promoters. The model predicted a higher promoter probability for all but one of the mutant promoters (p61:C>T) compared to the *TERT*

wild type promoter, predicting that 14 out of 15 known *TERT* promoter activating variants¹⁵ increase gene expression (**Extended Data Fig. 8a**). The model predictions also correlated well with previously published saturation mutagenesis MPRA experiments of the *TERT* promoter (**Extended Data Fig. 8b-d**), even when the CNN was trained for classification, not for regression. Training of a conventional regression model is not possible using the STARR-seq data as the size of the ultra-complex DNA library prohibits precise determination of initial input frequencies of individual sequences. However, the saturation mutagenesis experiments were carried out in different cell lines than GP5d for which our model was trained, but the correlation between the predicted and the measured effects of the variants was still close to the correlation observed between two experimental MPRA datasets using different cell lines.

Next, we used the CNN trained on the STARR-seq promoter sequences to predict the positions of active transcription start sites in the GP5d cells, as measured using CAGE (see **Methods**). The predictions were compared against a position-specific logistic regression model (see section **Position-specific logistic regression classification** in **Supplementary Methods**) of the STARR-seq promoters as well as against PWM and CNN models trained on the experimentally validated human genomic promoters downloaded from the Eukaryotic Promoter Database. For each model, we predicted the TSS probability by scoring each 120 bp long sequence within 500 bp from each of the active GP5d TSSs in the unseen test set. For each model and each test set TSS, the position giving the highest TSS probability was chosen as the predicted TSS position, meaning that for each model, one final TSS position prediction was made per each test set TSS. The rationale for this experiment was to see how well the models have learned features that determine the position of the TSS. As shown in **Fig. 6g**, CNN trained on random promoter data outperforms PWM-based models, regression models, and CNN trained on genomic promoter data in predicting active TSS positions in GP5d cells. The TSS probability of each position within ± 500 bp from the known TSS position was evaluated for each model, and the most likely TSS position was used as the predicted TSS. The score shown in **Fig. 6g** indicates the fraction of predicted TSS positions falling within ± 25 bp from the annotated TSS positions in the genome for each model separately.

Models trained on the STARR-seq promoters predict the TSS position in the genome more accurately than similar models trained on the genome itself, indicating that the training data with a better controlled background and more samples can allow learning more accurate models of gene regulatory elements (see also **Extended Data Fig. 8e**). Moreover, the STARR-seq CNN model outperformed the STARR-seq position specific logistic regression that uses as features the position-specific enrichment of the known TF binding motifs and promoter-specific features (such as TATA-box and Initiator, see **Supplementary Table 12**) in the training data, both in TSS position prediction (**Fig. 6g**) and slightly in classification (**Supplementary Fig. 4**). This indicates that similarly to the random enhancer CNN, the promoter capture CNN has also improved the motifs for better classification.

Mutual information (MI) analysis shown in **Extended Data Fig. 8f,g** visualizes the pairwise dependencies between position-specific 3-mer distributions learned by the STARR-seq promoter CNN and the genomic promoter CNN models, respectively (see section **Interpretation of convolutional neural network classifiers** in **Supplementary Methods** for details). These figures were generated from sequences that each of the models predict to represent highly active promoters, and thus they visualize the interaction patterns that each of the models looks for in sequences they deem as highly active promoters. The genomic promoter CNN mostly uses information right at the TSS (position 100 in **Extended Data Fig. 8f,g**), while the STARR-seq promoter CNN has learned a pattern that includes two other MI peaks before the TSS which is likely helping in discriminating between real active TSSs and other sequences in the genome.

The number of 5 bp long filters in the first layer of the CNN models was capped at 256, which with the minimum dropout of 0.3 allows the models to learn approximately 180 different 5 bp long filters representing binding motifs. These 5 bp long motifs can then be combined together to form representations of longer motifs (either continuous or gapped) by the subsequent layers of the network. In light of the motif matching analyses that show strong activity for only a limited number of motifs (**Fig. 5b**, **Fig. 6d**), and the good classification performance of the promoter CNN models (**Supplementary Fig. 4**), this size limitation of the CNN models is reasonable.

7) Enhancer-promoter interactions are additive and non-specific in nature

Fig. 7 utilizes the data generated from a binary STARR-seq experiment in which both promoter and enhancer elements comprise of random synthetic sequences. Here, the focus is on analyzing the interactions between promoters and enhancers in an unbiased manner.

We set out to test if a deep learning model is able to learn information that helps in classifying sequence pairs to active and inactive promoter-enhancer pairs from the specific pairing of the active promoter and enhancer sequences in the binary STARR-seq experiment. As machine-learning model performance is dependent on the model architecture and the hyperparameters, we planned an *in-silico* experiment where exactly the same kind of a convolutional neural network (CNN) model was trained on differently permuted datasets from the binary STARR-seq experiment. The CNN model consisted of two convolutional input bodies, one of which reads in the promoter sequence and the other the enhancer sequence. The number of dilated convolutional layers per body was optimized during training as described in the **Methods**. Before the prediction verdict, the information from these separate convolutional bodies was integrated with a fully connected layer to enable learning interactions between features in the promoter and the enhancer parts of the binary STARR-seq data.

In this *in silico* experiment, the original pairing from the binary STARR-seq experiment should contain information about any specific interactions between the specific pairing of promoter and enhancer sequences observed to be active in the STARR-seq experiment (denoted as “paired” in **Fig. 7c**). We then permuted the pairing of active promoters and enhancers so that each active promoter was still paired with an active enhancer, but with a different enhancer than in the original data (denoted as “permuted” in **Fig. 7c**). This permutation removes information about specific interactions in the promoter and enhancer pairs. Permutating the pairings between the promoters and enhancers resulted in similar performance (paired Student’s t-test two-sided P value = 0.134 for comparison of AU_{prc} from “permuted” CNN to “paired” CNN across the tested hyperparameters; **Fig. 7c**), indicating the absence of specific interactions between promoters and enhancers in our binary STARR-seq experiment.

In the “enhancer from input” and “promoter from input” training sets, the promoters and enhancers, respectively, were paired with a randomly sampled inactive sequence from the input library. In the “enhancer from input” model, the classification is purely based on the promoter features and in the “promoter from input” purely on enhancer features. We observed that both the promoter and the enhancer sequences contain information that is helpful in the classification task. When active promoter sequences were paired with inactive sequences from the input library (denoted as “enhancer from input” in **Fig. 7c**), the classification performance of the CNN model dropped significantly. An even clearer drop in classification performance was observed when active enhancers were paired with inactive sequences from the input library (denoted as “promoter from input” in **Fig. 7c**).

Supplementary Methods

STARR-seq reporter library construction and cloning

TF motif input DNA library

A pool of 92,918 oligos with a length of 79 nucleotides (nt) was designed with a 49-nt variable region and two 15-nt flanking regions with constant sequences for the library cloning (see Motif library design for more details), and synthesized by CustomArray Inc. The oligo pool was prepared for cloning in a two-step protocol using Phusion DNA polymerase (Thermo Fisher) and Oligos 1 and 2. First, 2.5 pmol of the oligo pool was double-stranded using 100 pmol of Oligo 2 in two parallel reactions [98 C for 3 minutes (min), followed by 5 cycles of 98 C for 10 seconds (s), 55 C for 15 s, 72 C for 15 s, and the final extension at 72 C for 2 min]. The two reactions were then split into ten reactions and after adding 10 pmol of the Oligo 1 to the reactions the PCR was performed for ten additional cycles using the same conditions. Ten PCR reactions were pooled and the 127-bp product was gel-purified. The pGL4.10-Sasaki-SS (a) and pCpG-free- EF1 α -SS (b) vectors were linearized by digestion with AgeI and SalI for 3 h at 37 C and gel-purified. For each backbone, the In-Fusion cloning (Clontech) was performed in 20 reactions using 200 ng of linearized vector and 50 ng of double-stranded oligo pool according to the manufacturer's instructions. Five In-Fusion reactions were pooled and purified using MinElute PCR purification columns (Qiagen) and eluted in 12.5 μ l nuclease-free water per column. The bacterial transformation was done by electroporation using Gene Pulser Xcell (Biorad) in 20 parallel reactions with 2.5 μ l of purified eluate and 20 μ l of E. coli 10G SUPREME (Lucigen) or Transformax EC100D pir-116 (Lucigen) electrocompetent cells for the pGL4.10-Sasaki-SS (a) and pCpG-free- EF1 α -SS (b) vectors, respectively, using the manufacturer's recommendations. To each electroporation cuvette, 1 ml of recovery media (Lucigen) was added, and the cells were incubated for 1 h at 37 C and 250 rpm. All cultures were then pooled and added to 5L of LB media with 100 μ g/ml ampicillin (pGL4.10-Sasaki-SS; a) or 25 μ g/ml zeocin (pCpG-free- EF1 α -SS; b) and grown overnight at 37 C and 250 rpm until the O.D. reached 1.0. The bacterial cells were harvested by centrifugation and plasmid DNA was isolated using EndoFree Plasmid Giga kit (Qiagen).

Genomic DNA input library

Genomic DNA was isolated from GP5d colon cancer cells using DNeasy Blood and Tissue kit (Qiagen) and treated with RNaseA (Thermo Fisher) followed by purification. The genomic DNA was fragmented to an average size of ~500 bp using Covaris S220 according to the manufacturer's recommendations. The fragmented genomic DNA (400 ng per reaction in ten reactions) was end-repaired, dA-tailed, and ligated to custom CpG-free annealed adapters (Oligo 3 - Custom CpG-free P7 adapter and Oligo 4 - Custom CpG-free P5 adapter; annealed according to the standard Illumina protocol). All adapter-ligated gDNA was purified and amplified for 10 cycles in 20 PCR reactions using KAPA HiFi master mix (Roche) and Oligos 5 and 6 which add the homology flanks for the NEB HiFi DNA assembly. The PCR product was pooled and purified using 0.8x volume of AMPure XP beads (Beckman Coulter) using the manufacturer's instructions followed by MinElute column purification. The pCpG-free-Sasaki-SS-v2 vector (d) was linearized using AflII and PvuII for 3h at 37 C and gel-purified. The PCR fragments were recombined to the vector in 50 parallel NEBuilder HiFi DNA assembly reactions (NEB) according to the manufacturer's instructions using 150 ng of linearized vector and 50 ng of custom CpG-free adapter-ligated genomic DNA. The reaction products were pooled (five reactions per column) and purified using MinElute columns (Qiagen) and eluted in water. The bacterial transformation was done in 50 reactions using 2.5 μ l of purified eluate and 20 μ l of Transformax EC100D pir-116 (Lucigen) by electroporation (Gene Pulser Xcell, Biorad) using the manufacturer's recommendations. To each electroporation cuvette, 1 ml of

recovery media (Lucigen) was added, and the cells were incubated for 1 h at 37 C and 250 rpm. The 50 cultures were then pooled and added to 6L of LB media with 25 ug/ml zeocin and grown overnight at 37 C and 250 rpm until O.D. reached 1.0. The bacterial cells were harvested by centrifugation and plasmid DNA was isolated using EndoFree Plasmid Giga kit (Qiagen).

Random enhancer oligonucleotide DNA input library

The random enhancer library was constructed from a 200-nt single-stranded Ultramer oligonucleotide (Oligo 7) harboring a 170-nt random sequence (170N) flanked by 15-nt constant sequences for library cloning. Double-stranded library was produced from 334 ng (5 pmol containing $\sim 3 \times 10^{12}$ molecules) of template oligo by employing a similar PCR strategy as described above for the TF motif library using Phusion DNA polymerase (Thermo Fisher) and Oligos 8 and 9 that introduce custom CpG-free sequencing adapters and flanking sequences homologous to the pCpG-free-Sasaki-SS-v2 vector (d). The NEB HiFi assembly between the random enhancer PCR product and the AflIII-PvuII fragment from the pCpG-free-Sasaki-SS-v2, as well as electro-transformation and plasmid DNA isolation were performed as described above for the genomic DNA library.

Random promoter and random enhancer oligonucleotide DNA input library

The random promoter-random enhancer library was constructed by using two 190-nt single-stranded Ultramer oligonucleotides (Oligos 10 and 11) with 150-nt random sequences (150N). Each oligo harbors two 20-nt constant sequences that facilitate the library cloning to the pCpG-free-promoter-enhancer-SS vector (e). First, the constant sequences at the 3' ends of the oligos anneal to the pCpG-free-promoter-enhancer-SS vector in a PCR reaction with Phusion DNA Polymerase (Thermo Fisher), amplifying the region between AgeI and SalI sites from the backbone. Then, the PCR product constituting the random promoter-random enhancer library (of size 555 bp; including 150-nt random sequences, the synthetic intron, and the small Dm tal-1A ORF) was cloned into the pCpG-free-promoter-enhancer-SS vector (e) linearized using AgeI-SalI using the constant sequences introduced by the 5' ends of the oligos. The NEB HiFi assembly, electro-transformation, and plasmid DNA isolation were performed as described above for the genomic DNA library.

STARR-seq reporter library and input DNA library construction

First strand cDNA synthesis was done with 2.5-5 ug of polyA(+) RNA and with Superscript III (Invitrogen, #18080-044) using a reporter-RNA specific primer (Oligo 15) in 10-20 reactions depending on polyA(+) RNA yield. This was followed by RNase A treatment for 1 h at 37°C and purification using MinElute PCR purification columns (Qiagen). cDNA amplification was performed with reporter-specific nested cDNA primers (Oligos 16 and 17 for libraries in vectors d and e, Oligos 16 and 18 for libraries in vector b, and Oligos 19 and 18 for libraries in vector a) using KAPA HiFi PCR Master mix (Roche) in the same number of reactions as done for the reverse transcription (98 C for 2 min, followed by 15 cycles of 98 C for 15 s, 65 C for 30 s and 72 C for 30-70 s). The PCR products were purified using 0.9X AMPure XP beads as per manufacturer's instruction followed by elution in nuclease-free water. The final PCR reactions to produce Illumina-compatible sequencing libraries were prepared from the entire amplified cDNA using KAPA HiFi PCR Master mix (Roche) at 98 C for 2 min, followed by 8-10 cycles of 98 C for 15 s, 65 C for 30 s and 72 C for 30 s. The primers used for different libraries are as follows: TF motif libraries in pGL4.10-Sasaki-SS (a) and pCpG-free-EF1 α -SS (b) vectors were amplified using standard Illumina Universal and index primers (NEB #E7335S) and sequenced using standard Illumina chemistry. Genomic DNA and random enhancer libraries in pCpG-free-Sasaki-SS-v2 vector (d) were amplified using custom CpG-free primers (Oligos 20 and 21) and random promoter-random enhancer libraries in pCpG-free-

promoter-enhancer-SS vector (e) using Oligos 22 and 21. All custom CpG-free libraries were sequenced using custom read 1 and read 2 primers (Oligos 23 and 24) and custom i7 index read primer (Oligo 25). For preparing sequencing libraries from the input DNA, plasmid DNA from each library design was amplified in ten parallel reactions (10 ng DNA per reaction) using Phusion DNA Polymerase (Thermo Fisher) as above. For TF motif libraries in pGL4.10-Sasaki-SS (a) and pCpG-free- EF1 α -SS (b) vectors, standard Illumina primers (NEB #E7335S) and sequencing chemistry were used, and for all the libraries in pCpG-free-Sasaki-SS-v2 (d) and pCpG-free-promoter-enhancer-SS (e) vectors, Oligos 20 and 21 were used for PCR amplification and Oligos 23-25 for sequencing. All libraries were sequenced either single-end or paired-end as per Illumina's standard instructions and protocols on suitable Illumina platforms like MiSeq, NextSeq500, HiSeq4000 and NovaSeq.

Template switch library preparation

Briefly, TurboDNase-treated RNA was incubated at 72 C for 3 min with a custom biotinylated STARR-seq specific RT-primer (Oligo 26) and dNTPs in two reactions having 25 ng of RNA per reaction, followed by immediate chill on ice. The first strand synthesis was performed using 100 units of Superscript IV RT (Invitrogen) in 1x SS-IV RT buffer containing 10 units of RNase OUT (Invitrogen), 5 mM DTT, 6 mM MgCl₂ (Sigma), 1M Betaine (Sigma) and 1 uM custom template switch oligo (Oligo 27) compatible with the custom CpG-free Illumina sequencing by incubating the reactions at 50 C for 15 min followed by 80 C for 10 min. cDNA was purified using AMPure XP beads (Beckman Coulter) and one cDNA reaction product was split into two for PCR amplification using KAPA HiFi master mix (Roche) together with USER enzyme (NEB) and Oligos 28 and 17 for enrichment of STARR-seq reporter-specific template (98 C for 3 min, followed by 15 cycles of 98 C for 20 s, 67 C for 15 s, 72 C for 6 min and final extension at 72 C for 5 min). The PCR product was purified using AMPure XP beads (Beckman Coulter) followed by a second PCR for a total of 5 cycles for Illumina library preparation using custom CpG-free Oligos 20 and 21. The final library was purified and sequenced using Oligos 23-25 on NextSeq and NovaSeq platforms.

Chromatin immunoprecipitation (ChIP-seq) and gene expression analysis (RNA-seq)

For ChIP-seq, fresh formaldehyde-crosslinked chromatin from the cells was used to immunoprecipitate DNA using Dynal-bead coupled antibodies, followed by standard ChIP-seq library preparation for Illumina sequencing. The libraries were single-read sequenced on HiSeq4000 and NovaSeq6000. The sequencing reads were aligned to the human genome (hg19) using bowtie2¹⁷ (version 2.2.4) and peak calling (narrow peaks for TF ChIP-seq and activating histone marks and broad peaks for repressive histone modifications) was performed using MACS2¹¹ (version 2.1.1) using default parameters. Super-enhancers for GP5d cells were calculated using SMC1 and H3K27ac ChIP-seq data using the ROSE pipeline¹⁸ (version 0.1). The peak files were filtered for the ENCODE blacklisted region (accession ENCSR636HFF) before further downstream analysis.

For gene expression profiling, total RNA was isolated using RNeasy Mini kit (Qiagen) with on-column DNase I treatment from the following conditions in three biological replicates: HepG2 cells with mock treatment (DMSO), with 350 μ M 5-FU treatment, and after transfection of the genomic STARR-seq library using similar conditions as in the STARR-seq experiments described above at 24 and 48 h time points; GP5d cells 24 h after genomic STARR-seq library transfection. RNA-sequencing libraries were generated from 1 μ g of total RNA samples using KAPA stranded mRNA-seq kit for Illumina (Roche) as per manufacturer's instruction and single-read sequenced on NovaSeq 6000 (Illumina). These data sets were used for analyzing the effect of plasmid transfection on cellular alarms by comparing gene expression between different treatments in HepG2 cells at 24 h time point (since the same time

point was used in the STARR-seq experiments), and for mapping the differential expression of TFs between HepG2 and GP5d cells. The transcript level counts were estimated with kallisto¹⁹ version 0.46.1 in the strand-specific mode (option --rf-stranded) using Ensembl transcript annotation (Ensembl GRCh37 release-101 cDNA file). Differential expression analysis was performed with sleuth²⁰ version 0.30.0. Each treatment at different time points was treated as a separate condition, and the conditions were put into two models, one for the HepG2 treatments and another for the cell line comparison. Then differential expression was estimated by comparing individual conditions to a control condition (mock control when testing different HepG2 conditions and HepG2 expression when comparing two cell types) using the wald test. For gene set enrichment analysis (GSEA), gene lists for p53 and interferon signaling pathways were obtained from the Molecular Signatures Database (MSigDB v7.4, release c2.cp.v7.4.symbols.gmt) and preranked analysis using GSEA (version 4.1.0) was ran on the list of differentially expressed genes (preranked according to log₂ fold-change from STARR-seq transfection vs. mock and 5-FU vs. mock) against these signatures. For comparing TF expression levels to motif activities in STARR-seq TF motif library in GP5d cells, the transcript abundances (transcripts per million; tpm) for three replicate samples were taken from RNA-seq data from ref. ²¹. The expression of each TF in GP5d cells was summarized by taking the mean expression over the replicates for its most highly expressed transcript.

Chromatin accessibility (ATAC-seq)

The ATAC-seq libraries were prepared from 50,000 cells as previously described²² for GP5d cell line and for HepG2 cells collected 24 h after the following treatments: mock (DMSO), 350 μ M 5-FU treatment, and transfection of the genomic STARR-seq library using similar conditions as in the STARR-seq experiments described above. The cells were washed in ice-cold PBS and resuspended in 50 μ l of lysis buffer and incubated for 10 min on ice. The pellet was lysed and transposed with Tn5 transposase in 2X tagmentation buffer (Illumina kit) and incubated for 30 min at 37 C. The reaction was purified using a MinElute purification kit and eluted in nuclease-free water. The samples were amplified for 5-8 cycles as determined by qPCR for Illumina sequencing using Nextera library preparation kit (Illumina) and samples were paired-end sequenced on HiSeq4000. The samples were demultiplexed and paired-end fastq files were processed using an in-house pipeline comprising of TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), BWA aligner²³, Picard (<http://broadinstitute.github.io/picard/>) and broad-peak calling by MACS2¹¹. The peak files were filtered for the ENCODE blacklisted regions as described earlier. In the genome browser snapshots, the traces from BAM coverage files are shown.

Active transcription factor identification (ATI) assay

The assay was performed *in vitro* by mixing 5 μ l nuclear protein extracted from GP5d cells (2 μ g/ μ l), 5 μ l 140 bp double stranded DNA (dsDNA) oligos³ containing 40 bp random sequence in the middle (10 pmol), and 5 μ l 3 $\times$ protein binding buffer (420 mM KCl, 15 mM NaCl, 3 mM K₂HPO₄, 6 mM MgSO₄, 300 μ M EGTA and 9 μ M ZnSO₄, 60 mM HEPES, pH = 7.5) and incubating for 30 min at room temperature. The poly-dIdC was supplemented in the reaction (5 ng/ μ l final concentration) to decrease non-specific binding. Electrophoretic mobility shift assay (EMSA) was then conducted using commercial DNA Retardation Gel (Invitrogen, #EC63652BOX) in 0.5 $\times$ TBE buffer (1 mM EDTA in 45 mM Tris-borate, pH 8.0) at 106 V voltage for 70 min. The gel above the 300 bp DNA marker was collected, eluted in 300 μ l Tris buffer (10 mM Tris-Cl, pH 8.0) and incubated at 65 °C for 3 h. The eluted DNA was amplified with Phusion polymerases (Thermo Scientific, #F530L); 4 pmol of each primer were used for the amplification. Before the final step of amplification, the same amount of primers was added to convert the remaining single stranded DNA (ssDNA) to dsDNA. The

amplified DNA library was incubated again with an aliquot of the same protein extract as above and the whole process was repeated for three more times. The PCR products from different cycles of ATI were purified and sequenced by Illumina Hiseq4000. The preprocessing of the data was done as described earlier in ref. ³. The matching of known motifs was performed in the same way as for all other experiments (described below in **Matching of known motifs**).

Transient transcriptome sequencing (TT-seq)

Transcribed enhancer regions defined using TT-seq data are based on ref. ²⁴. Briefly, TT-seq from GP5d colon cancer cells was performed in two biological replicates as described²⁵. TT-seq libraries were sequenced to a depth of ~120 million uniquely mapped paired-end reads and data analysis for identification of genomic intervals corresponding to continuous uninterrupted transcription (defined as transcription unit, TU) was performed using GenoSTAN²⁶. TUs were classified into two groups: those overlapping with annotated protein-coding genes were defined as mRNAs and remaining as non-coding RNAs (ncRNAs). Putative enhancer RNAs (eRNAs) were further subclassified using histone modification patterns by defining a large set of putative enhancer regions from the publicly available datasets covering the non-coding regulatory genome^{27,28}. eRNAs were further defined from the pool of ncRNAs on the basis of three criteria; first, origin should overlap within TSS $\pm$ 500 bp (enhancer region), second, it should be outside of TSS $\pm$ 1 kbp (promoter region), and third, transcription is bidirectional as measured with TT-seq. This annotation led to identification of 6774 enhancer regions in GP5d colon cancer cells. The annotated enhancers were filtered for blacklisted regions as described before and also for chromosomes other than 1-22 and X.

Motif collection

For testing activities of known TF motifs, a set of 3226 HT-SELEX motifs were collected (refs ^{10,29,30} and unpublished draft motifs). A more compact set of motifs representing different binding specificities was generated by first constructing a dominating set (880 PWMs) covering motifs from the above sources using the same method and motif distance threshold as in ref. ²⁹. Then, in order to retain information of TF binding differences between methylated and non-methylated DNA ligand¹⁰, for each methyl (or non-methyl) motif in the dominating set, the closest non-methyl (methyl) motif of the same TF was added to the representative set (respectively) if it was not yet in the set. This resulted in a representative set of 1121 HT-SELEX motifs (**Supplementary Table 3**). In cases where HT-SELEX motifs for several TFs are highly similar, the motifs have been named in figures according to TF class or subclass, in order to highlight that we do not know which of the TFs that have similar motifs binds to the motif in the cells. Same principle has been applied also when specificities of closely related TFs have not been measured and thus can reasonably be expected to be similar. **Supplementary Table 5** shows the naming for the motifs in each figure. A control set of reversed but not complemented motifs was generated by reversing the column order of each motif matrix. Additionally, the following promoter core motifs were collected for TSS analyses from literature: TATA box, Initiator, CCAAT-box, GC-box from ref. ³¹, BRE, MTE, DPE from ref. ³², and BANP from ref. ³³.

Motif library design

The synthetic oligo library design contained the 1121 HT-SELEX motifs in various sequence patterns. A pattern is defined as the combination and number of the motif consensus sequences, their relative orientation and spacings, and positions of degenerate N bases. Each of 727 monomeric and homodimeric motifs was included in the following patterns: consensus and its reverse complement (if palindromic added twice), reversed but not complemented control, each position of consensus at a time replaced with a degenerate base N, and two and

three copies of the consensus sequence in different orientations and spacings (three copies only for the motifs shorter than fifteen bases). Putting a degenerate base N at every position (in total 10,041 positions) generated in total 30,123 mutant consensus sequences.

In the patterns containing two and three copies of the consensus, the most defined position of the motif (position with maximum probability for any base in any position) was replaced with N. The two copy patterns included three relative orientations of the consensus C and its reverse complement R (CC, CR, and RC) and three copy patterns included four relative orientations (CCC, CCR, CRC, RCC). In the case of two copies, each orientation was included with all gap lengths from zero to six bases between the two copies. In the case of three copies the gap length was varied from zero to four bases (oligo length permitting) but the same gap length was used for both gaps in one pattern. Also, the consensus and reverse complement of the 394 heterodimeric motifs³⁴ was included. A subset of 245 heterodimeric motifs were also cut into two half-sites and the half-sites were added in the same patterns as two copies of a monomeric motif.

Each of the 43,251 motif patterns was embedded in two different sequence contexts. The contexts were chosen from two human genomic loci (context 1, chr10:77103489-77103535 and context 2, chr8:21525556-21525602, in hg19 coordinates) that do not contain high affinity sites of known motifs. Finally, 2-6 bases long random sequence (UMI) was put to the 5' end of the sequence to create an approximately uniform base distribution for sequencing. Both context sequences were also included alone with five different UMI lengths. Thus, in total 86,512 sequences were used for testing TF activity. The sequences and their embedded motif patterns are given in **Supplementary Table 4**. The remaining 6,406 oligos from the total 92,918 sequence patterns were composed of 3,576 SELEX nucleosome bound and unbound sequences, 1,182 draft HT-SELEX models for RNA binding proteins³⁵ and 1,648 for tiling genomic regions corresponding to enhancer regions for MYC and CCND2 (**Supplementary Table 4**).

Enhancer activity of TF motif consensus sequences

For each TF consensus pattern, the reads containing the pattern were counted separately for the two sequence context and counts less than five in RNA and input DNA together were discarded from further analysis. The fold-change between RNA and input DNA was estimated using the function `PsiLFC` in R package `lfc` version 0.2.1³⁶ for each pattern in each context. To summarize the activity of one, two, or three copies of the motif, the median fold-change of all the patterns in both contexts containing the given number of the consensus sequences was used. For an individual consensus sequence, this included the patterns containing it or its reverse complement in both contexts. In the case of two or three copies, all consensus spacings and orientations were summarized together. The average fold-changes over all motifs in one, two, three copies were only calculated from those that could be detected with all copy numbers. If several sites acted without synergy, we assumed $\log_2$ fold-changes to grow linearly as a function of the number of sites. The motifs representing heterodimers were excluded from the analysis.

Analysis allowing base substitutions and generation of activity position weight matrix

For each pattern in a sequence context, the number of matching reads was counted both for the consensus sequence and its variations containing the designed base substitutions. The patterns detected at least 100 times in each context in RNA and input DNA together were considered. The $\log_2$ fold-change of a motif pattern in a context was estimated from the RNA and input DNA counts using the function `PsiLFC` in R package `lfc`. The activity PWMs were generated using the counts of the consensus sequence and all its single base substitutions. For each position of the consensus, the number of times each base was observed at that position in

the sequences otherwise matching the consensus was counted both in RNA and input DNA. Then such matrices were found that selecting from the input DNA sequences with them would generate the observed nucleotide distributions in STARR-seq RNA. In more detail, for each column independently a multinomial distribution was sought such that

$$\begin{aligned} sa_A b_A &= o_A \\ sa_C b_C &= o_C \\ sa_G b_G &= o_G \\ sa_T b_T &= o_T \\ a_A + a_C + a_G + a_T &= 1 \end{aligned}$$

where

- a_N is the probability of the nucleotide N in the multinomial distribution
- b_N is the count of the nucleotide N in the input DNA
- o_N is the observed count of the nucleotide N in STAR-seq RNA
- s is a size factor to account for the different sequencing depths

Motif library complexity

Based on the sequencing of the pCpG-free-EF1 α -SS input library, the motif library was estimated to contain approximately 26.9×10^6 distinct sequences when taking into account different UMIs (corresponding in total 1.3×10^9 bp), read counts are given in **Supplementary Table 7**.

Genomic library complexity analysis

Genomic STARR-seq input DNA library complexity was estimated using the preseq program³⁷ (version 2.0.0) `lc_extrap` tool that estimates how many distinct fragments would be observed based on reads in an initial sample if a given number of reads was sequenced. The mapped paired-end reads (bam) were given as input and the expected yield of 2.09×10^9 distinct fragments (assuming 2×10^{10} reads were sequenced) was used as the complexity estimate (10^{12} bp). Thus, the library was estimated to cover the human genome (3.2×10^9 bp) with 1.53 bp resolution on average. The resolution of STARR-seq output fragments in highly active genomic regions was broadly consistent with the high input resolution, taking into account that the fragment activity depends on which part of the regulatory element it covers.

Random library complexity and information analysis

The complexity of the random enhancer STARR-seq input DNA library was estimated by first creating a robust set of sequences originating from the same clones and their counts. This was done by clustering a randomly sampled set of input sequences using `starcode`³⁸ (version 1.3) so that first reads with edit distance four or less were connected and then reads in the same connected component were put to one cluster. The sizes of the sequence clusters were then given as input to `preseq` resulting in estimated 2.4×10^9 distinct sequences (corresponding in total 4.1×10^{11} bp). The same approach gave an estimate of 0.9×10^9 promoter sequences (1.4×10^{11} bp) and 1.2×10^9 enhancer sequences (1.8×10^{11} bp) in the binary STARR-seq input library. To confirm that these extremely complex libraries are effectively transfected to the cells, we estimated the number of plasmid copies per cell based on the comparison of read coverage between plasmid and genomic DNA from a control ChIP-seq experiment using a non-specific IgG antibody. This analysis revealed over 2500 plasmid copies per cell and based on this we estimate that each distinct random enhancer sequence was transfected to cells over 500 times on average.

To compare to the genomic conservation, we assumed that a TF motif typically has ~15 bits of information content. As it can be placed in ~320 positions in a 170 bp long random enhancer, one TF binding site contributes approximately $15 - \log_2(320) = 6.7$ bits of

information (see ref. ³⁹). Thus, a site would correspond to approximately seven conserved bases (> 1 bit of information).

Genomic STARR-seq analysis

First, demultiplexed Illumina STARR-seq RNA and input DNA paired-end reads (trimmed to common length 2×37 bp) were aligned to the human genome (hg19) using bowtie2¹⁷ (version 2.2.4, option --maxins 1000). Before peak calling the mapped read pairs were deduplicated using picard (version 2.9.0) and paired-end reads that had mapping quality <20 or mapped in a discordant orientation were discarded using samtools version 0.1.19. MACS2¹¹ version 2.1.1 was used to call peaks in paired-end mode (-f BAMPE) so that the fragment endpoints were inferred from alignment results. Input DNA was used as control in peak calling and the called peaks were filtered for the ENCODE blacklisted regions (accession ENCSR636HFF) before subsequent downstream analysis. For masking repeats, a RepeatMasker file from UCSC table browser (for hg19) was used.

All STARR-seq RNA fragments from each cell line were used in peak calling (**Supplementary Table 7**) unless otherwise stated. Peaks were also called separately from two replicates in HepG2 cell line with 3295 peaks overlapping out of 6414 and 7376 peaks. IDR software⁴⁰ (version 2.0.3) was then used together with combined sample peaks to call high confidence peaks (2186 peaks with IDR < 0.1). In GP5d cells, genomic STARR-seq analysis was performed in two sublines (p53 wt and null) under two conditions (methylated or not), but due to the very large size of the experiments, replicates were not included for each condition. To enable calling reproducible peaks from the GP5d data, we utilized an internal control approach wherein two sets of peaks are built from a single replicate by splitting the fragments to two sets based on their mapping to either even or odd positions of the genome. These “*in silico*” replicates were then used for IDR analysis resulting in 1970 and 3250 high confidence *in silico* peaks (IDR < 0.1) in HepG2 and GP5d wt cells, respectively. Comparison of the high confidence peaks from biological and *in silico* replicates in HepG2 cells revealed that this IDR method yields similar peak-calls (~90% specificity if biological replicate analysis is considered ground truth; see **Extended Data Fig. 5a**). Together, these analyses show the high reproducibility of the strong genomic STARR-seq peaks.

Genomic feature overlap analysis

Overlaps between the GP5d genomic STARR-seq and other genomic features were calculated from peaks called with MACS2¹¹, with the exception of the TT-seq enhancers for which the estimated enhancer regions were used. Only chromosomes 1-22 and X were used in the analysis. All overlaps between the peaks were calculated using Bedtools⁴¹ (version 2.25.0). The motifs used in calculating the matches to STARR-seq peaks are listed in **Supplementary Table 5**. For comparison of STARR-seq peak overlap with ATAC-seq, H3K27ac ChIP-seq, and TT-seq enhancers (**Extended Data Fig. 5c**, left), Fisher’s exact test two-tailed *P* values were calculated using “bedtools fisher”⁴¹ after filtering out the positions in the ENCODE blacklist (peaks hitting these regions would not be considered). Note that in most cases the overlaps are much larger than expected purely at random leading to *P* values that are too small to represent as floating-point numbers. These cases are marked as $P < 2.2251 \times 10^{-308}$.

Genomic STARR-seq in HepG2 cells was compared with data downloaded from the ENCODE project: ATAC-seq (ENCSR042AWH, replicate 1), histone modification ChIP-seq experiments for H3K27ac (ENCSR000AMO), H3K27me3 (ENCSR000AOL), and H3K9me3 (ENCSR000ATD), and H3K4me1 (ENCFF424GUI), as well as ChIP-seq data sets for TP53 (ENCSR980EGJ), MED1 (ENCFF493UFO), and MED13 (ENCFF003HBS). The IDR-thresholded peaks and bigWig signal files showing fold-change over control generated from all replicates were used except for ATAC-seq and TP53 ChIP-seq. The reads for ATAC-seq

replicate 1 were reanalyzed in hg19 coordinates in the same way as GP5d ATAC-seq data resulting in 60,500 peaks. For TP53 ChIP-seq, the ENCODE peaks were lifted over from GRCh38 to hg19 coordinates and the reads for replicate 1 were remapped to hg19 and MACS2 was used to generate a normalized coverage file. For the rest of the TFs and other chromatin-associated proteins, the ChIP-seq peaks were taken from ref.⁴² (GEO accession GSE104247), and the corresponding bigWig files were downloaded from the ENCODE portal. Super-enhancers for HepG2 are from <http://www.lipathway.net/sedb>. The overlaps between different features were calculated using bedtools (chromosomes 1-22, X and Y). The Euler (**Fig. 4a**; R package eulerr) and bar (**Extended Data Fig. 6a**; R package UpSetR) diagrams show other features overlapping the top quartile of all ATAC-seq and STARR-seq peaks according to maximum fragment coverage. When calculating overlaps between chromatin-associated proteins in open regions with or without STARR-seq signal (**Extended Data Fig. 6a**), only STARR-seq peaks with IDR < 0.1 calculated from two STARR-seq replicates were used. The PolIII-associated proteins POLR2AphosphoS2, PAF1, POLR2A, ZC3H4, TBP, POLR2AphosphoS5, and SSRP1 were excluded from this overlap analysis resulting in 202 proteins. In the overlap analysis, all Ensembl TSS (GRCh37, release 101) extended 1 kb to both directions were used.

***De novo* motif mining**

De novo motif mining for the peaks called from ATAC-seq, ChIP-seq and genomic STARR-seq were performed using HOMER⁴³ (version 4.10.3). Sequences used for motif mining of different enhancer classes in HepG2 cells were based on the intersections as in Euler/UpSet diagrams in **Fig. 4a**; **Extended Data Fig. 6a**, with the overlaps calculated against top quartile of the ATAC or STARR-seq peaks, respectively. In addition, the sequences overlapping TSS-regions (from Ensembl GRCh37, release 101, extended 1 kb to both directions) were excluded, resulting in 1,524 closed chromatin enhancers, 971 classical enhancers, and 3,797 chromatin-dependent enhancers that were used in the analysis. For the sequences enriched from the random enhancer and random promoter-random enhancer STARR-seq experiments and from the ATI assay, *de novo* motif mining was done using the “Autoseed” program³⁰ as described earlier^{3,30}. From the random enhancer STARR-seq in GP5d cells, the sequences were cut to 40 bp long non-overlapping subsequences starting from position 6 and ending at position 165, and the subsequences containing N were removed (see **Supplementary Table 7** for the numbers of analyzed subsequences). “Autoseed” program³⁰ was also used to mine *de novo* motifs enriched at specific positions in relation to TSS using TSS-aligned sequences from GP5d cells (see section “Mapping TSS positions based on template switching”) and input DNA sequences sampled from the same positions (**Supplementary Table 7**). The resulting seven *de novo* motifs were mined from the subsequences spanning the following positions in relation to TSS: two initiator motif variants from position one at TSS (only forward strand), CREB motif and two CREBMAF heterodimer variants from -30 to 30, and TATA box promoter from -30 to 23. These motifs were also included to motif enrichment comparison between promoter and enhancer sequences.

In addition to the “Autoseed” analysis, we performed *de novo* motif discovery using STREME⁴⁴ program (version 5.3.3) from the common 371,390 enhancer sequences that were observed in both replicates from random enhancer STARR-seq in GP5d cells compared to randomly sampled input sequences. Due to the large size of the data set, only one seed per each motif discovery round was optimized using the iterative refinement algorithm and half of the whole data was used as the hold-out set to accurately estimate *P* values also for rare but highly enriched motifs (options --hofract 0.5 --nref 0 --minw 6 --maxw 20). The discovered significant motifs (*P* value < 0.05) were compared to known TF motifs using TOMTOM⁴⁵ web-service (version 5.3.3) with default options.

Conservation of genomic STARR-seq elements

Conservation of genomic STARR-seq peaks and input fragments was analyzed by calculating their average GERP scores⁴⁶ using precomputed base-wise GERP scores (hg19) from ref. ⁴⁷ (<http://mendel.stanford.edu/SidowLab/downloads/gerp/>). In the analysis, only chromosomes 1-22 were included, and the elements overlapping ENCODE blacklisted regions (ENCFF419RSJ) and UCSC RepeatMasker⁴⁸ “Repeats” track were discarded. A base pair was deemed conserved if its GERP score was higher than the average GERP score (~2.2) of the coding sequence⁴⁷. Additionally, the GERP scores were calculated for three known enhancers as detailed below.

- GP5d genomic STARR-seq peaks, mean GERP score = 0.14. The average number of conserved base pairs in 170 bp surrounding the STARR-seq peak summits was ~50.8 (~169.4 for whole peaks, corresponding to ~42.6 when the average width of a STARR-seq peak, ~675.5 bp, is scaled to 170 bp).
- 100,000 randomly sampled genomic STARR-seq input fragments, mean GERP score = 0.01.
- The MYC335 enhancer (chr8: 128413174-128414429)⁴⁹, mean GERP score = 3.07, number of conserved base pairs = 921.
- The SHH enhancer (chr7: 156583796-156584568)⁵⁰, mean GERP score = 3.66, number of conserved base pairs = 642.
- The Sox9 enhancer (chr17: 69480826-69481362)⁵¹ with respective coordinates for human SOX9 enhancer (hg19) obtained using UCSC genome LiftOver tool (<https://genome.ucsc.edu/cgi-bin/hgLiftOver>), mean GERP score = 0.70, number of conserved base pairs = 230.

Matching of known motifs

The motifs were matched to sequences using MOODS⁵² (version 1.9.3). The matching was done separately for each strand using strand- and sample-specific nucleotide frequencies so that the background probability of a match was 10^{-6} unless otherwise stated. When calculating motif matches for logistic regression PWM features, affinity threshold of 2 was used instead of a background probability threshold. Motif matches that resulted in occupancy probabilities smaller than 0.01 were discarded (see Logistic regression classification for details).

To determine the activity of an individual motif, the total number of its matches in the sequences was counted so that overlapping matches in different strands were counted only once. Then fold-changes between the motif match counts in RNA and a randomly sampled subset of input DNA were estimated using the function PsiLFC in R package lfc. This approach together with sample-specific motif matching effectively eliminated the effect of differing mononucleotide frequencies between samples. For random enhancers in GP5d cells the Pearson correlation between the percentage and the number of G and C in motif consensus and its enrichment for the most active 10% of motifs were -0.15 and -0.05, respectively, and for all motifs 0.17 and 0.15, respectively. When comparing two different RNA samples (for example two replicates), two different random samples of input DNA were used to avoid overestimating similarities by using identical input counts. To estimate the effect of the number of binding sites in the same sequence, the number of sequences having exactly two non-overlapping motif matches was counted, and the fold-change was compared to the fold-change of those having exactly one match. If the site occurrences are independent of each other, the expected frequency of several sites is the product of the individual frequencies. Thus, the expected $\log_2$ fold-change assuming independent actions of several motif occurrences was calculated as the sum of their individual $\log_2$ fold-changes.

Analysis of motif spacing

For the motif spacing analysis in STARR-seq random enhancers, the most enriched 10% of the motifs in GP5d cell line were chosen (73 motifs as heterodimer motifs excluded, $\log_2$ fold-change of the chosen motifs was over 0.19 when matching done with P value cut-off 10^{-6}). For the chosen motifs, the matching was done with a relaxed P value cut-off 10^{-4} against pooled sequences from both GP5d replicates and from each enhancer sequence only the strongest match for each motif was taken (so pairs of the same were not considered). For each motif pair, all overlapping motif matches were removed and for the remaining motif match pairs the distances between motif match information content centers were calculated and pairs having distance over 30 bp were discarded (the example cases shown in **Fig. 2c** were re-analyzed also with the maximum distance 50 bp shown). For each of 5,256 pairs (including the same motif pair in both orders), each configuration (orientation of the motifs relative to each other and distance between the information content centers) was separately tested for enrichment using one-sided Fisher's exact test to see if there was an association between the two classifications i) the particular spacing and orientation vs. all others and ii) motif matches in STARR-seq RNA vs. input DNA. The individual P values were corrected for the number of configurations tested for the pair using Holm's method. For 46 pairs the false discovery rate of the best configuration was < 0.1 (Benjamini Hochberg method used to correct for the total number of tested motif pairs). The p53-family motif match spacing with another match of itself was analyzed in the same way except that several matches of the motif in a sequence were considered.

The statistical power to detect specific motif spacings and orientations was analyzed by generating the same number of simulated pairs as there were tested pairs and then for each simulated pair the same number of configurations (n) as there were on average per tested pair. The input counts of the different configurations were then generated from the multinomial distribution so that each configuration had a success probability $1/n$ and that the total count matched the average DNA input count of a tested pair given the motif match threshold. Then for each simulated true positive pair with the fold-change f , the STARR-seq RNA counts were generated from the multinomial distribution so that one configuration had a success probability f times the probability of the other configurations (null hypothesis false). For the rest of the simulated pairs (null hypothesis true), the RNA counts were as for the input using probability $1/n$ for all configurations (null hypothesis true). In both cases the total count was set to the average STARR-seq count of a tested pair. Statistical testing was done for the generated counts as for the tested cases. Finally, for each fold-change, the proportion of the simulated true positive pairs for which the null hypothesis was rejected with a false discovery rate < 0.1 was reported as the power of the test.

Mutual information (MI) analysis

The binding events on the aligned STARR-seq reads (60+60 bp surrounding TSS from the template switch data) were analyzed by calculating the mutual information (MI) between 3-mer distributions at two non-overlapping positions of the aligned sequences. MI can be used to capture binding events, because if the binding contacts two continuous or spaced positions (3-bp wide) on the sequences at the same time, correlations will be observed for the 3-mer distributions at the two positions. Subsequently, the biased joint distribution will be detected as an increased MI between the positions. For two non-overlapping positions (pos1, pos2), the MI between them was estimated as described in ref. ⁵³. The calculation uses the observed frequencies of a 3-mer pair (3+3-mer), and of its constituent 3-mers at both positions:

$$MI(pos1, pos2) = \sum P(3+3\text{-mer}) \log_2 \frac{P(3+3\text{-mer})}{P_{pos1(3\text{-mer})} P_{pos2(3\text{-mer})}}$$

where $P(3+3\text{-mer})$ is the observed probability of the 3-mer pair (i.e. gapped or ungapped 6 mer). $P_{pos1}(3\text{-mer})$ and $P_{pos2}(3\text{-mer})$, respectively, are the marginal probabilities of the constitutive 3-mers at position 1 and 2. The sum is calculated over all possible 3-mer pairs using 17,235 TSS sequences. When calculating the positional 3-mer and the pairwise 3+3-mer distributions, a pseudocount of 10 was added to each k-mer. Pseudocount was used to alleviate the effects of estimating MI from a finite sample of sequences. Using pseudocount means assuming that when the observed sample count of a k-mer approaches zero, the estimated probability of observing this k-mer approaches a non-zero constant (instead of zero without a pseudocount). Reasonable changes in magnitude of pseudocount only affect the scaling of values of MI, not the position or shape of dependencies highlighted. Mean mutual information between 3-mer distributions separated by specific distances was computed by taking the mean of each diagonal of the mutual information matrix computed as described above.

Data preprocessing for machine-learning analysis

The datasets used in each machine-learning analysis and their division into training, test and validation sets are detailed in **Supplementary Table 7**. To enable sequences from genomic measurements (genomic STARR-seq and ATAC-seq) to be scored on the CNNs that were trained on the random enhancer STARR-seq data and vice versa, the length of the sequences fed to these models was standardized to 170 bp. Thus, additional preprocessing specific to machine-learning analyses was done for the genomic STARR-seq and ATAC-seq data.

First, an extended blacklist file was created to remove possibly problematic genomic regions that might cause the machine-learning models to learn biases instead of real signals. In addition to the standard ENCODE blacklist (ENCFF419RSJ), this extended blacklist contains all positions $\pm 1\text{Mb}$ from centromeres, all positions with Ns in the hg19 reference genome and non-uniquely mapping regions that were defined as follows: All unique 55-mers present in the hg19 reference genome were fetched and aligned back to hg19 reference with bwa aln²³ algorithm (version 0.7.15-r1142-dirty). Then each position that was not covered by reads mapped with MAPQ>20, was added to the extended blacklist. This extended blacklist covers around 12% of the hg19 reference genome.

GP5d genomic enhancer fragments were created by fetching the sequence (hg19) that maps between the paired end reads. The signal set (class 1) sequences were created by taking the 170 bp closest to the peak summit from each genomic STARR-seq fragment that overlaps with a GP5d genomic enhancer STARR-seq peak. Balanced control sets of 170 bp sequences (class 0) were sampled from the genomic STARR-seq input requiring that the reads map and do not overlap with regions covered by the extended blacklist or with GP5d genomic enhancer STARR-seq peaks. To ensure that the classifier does not learn any features possibly correlating with different input library coverage between the class 1 and class 0 sequences, the class 0 sequences were sampled in such a way that their input library coverage histogram matched the input library coverage histogram of the class 1 sequences. Input library coverage of each class 1 and class 0 sequence was calculated by counting how many input library fragments each of them overlaps. Then 36 evenly sized bins were created so that the first bin included coverages between 0 and 9 and the last between 350 and 359 (354 was maximum coverage for class 1 sequences). Then class 0 sequences were sampled so that their count in each bin equaled the count of class 1 sequences in that bin for each set (training, test, validation) separately. Sequences from random STARR-seq experiments were not mapped to any reference sequence at any point, so this precaution is not relevant with random STARR-seq experiments.

The GP5d ATAC-seq single-end short reads were extended to the average fragment size of the library (300 bp) by adding 300-readlen to 3'-end of each read (where readlen is the length of each read). For the class 1 signal set, extended fragments that overlap with any ATAC-seq peak were selected and the 170 bp sequence closest to the overlapping peak summit

was retrieved from the fragment. Exact duplicate sequences were discarded. A balanced negative set (class 0) was created by sampling random 170 bp sequences from the genome and not allowing them to overlap with ATAC-seq peaks or regions covered by the extended blacklist.

Logistic regression classification

The logistic regression classifiers were implemented using the LogisticRegression function from scikit-learn (version 0.21.3) library⁵⁴. All logistic regression models were regularized with L1 norm (LASSO). Using L1 norm as regularization is important for the interpretability of the model coefficients, as the set of PWMs used as features of the model (**Supplementary Table 10**) contains several matrices that can be very similar to each other. Thus, a non-regularized model, or a model regularized with L2 norm (ridge), could split effects into coefficients of similar PWMs. Using the L1 norm that penalizes solutions with a higher number of non-zero coefficients enables finding the best performing model with the lowest number of individual PWMs contributing to the model. Regularization with the L1 norm is known to perform well with correlated features given that the regularization strength is properly adjusted⁵⁵. The optimal regularization strength was chosen based on the area under precision-recall curve on the validation data (see **Supplementary Tables 8, 9** for the tested and final values, respectively). Classification performance on unseen test data is shown for each trained hyperparameter combination in **Supplementary Fig. 4**. Otherwise the regression was run on default parameters. First, a logistic regression classifier was trained using only features that count matches of individual PWMs (880 features, **Supplementary Table 10**). After this, a more complex classifier was fit with additional features counting all self-pairs ($A_i + A_i$, i runs over all the 880 PWMs), and all pairs of the top 20 strongest individual features (20 features with largest absolute value of the regression coefficient) from the simpler model (**Supplementary Table 11**) with all other PWMs ($S_j + A_i$, i runs over all the 880 PWMs, j runs over the top 20 PWMs from the simple model of 880 features). Thus, this more complex model is expected to cover all meaningful general pairwise interactions between the binding motifs of HT-SELEX PWMs. We also trained simple logistic regression models using the set of 22 *de novo* binding motifs discovered with STREME⁴⁴ (see **Supplementary Fig. 2a**) as features.

For each feature (corresponding to a PWM, X), the probability that a given read is occupied by a given TF or TF-pair is calculated by using an approach derived from ref. ⁵⁶, where:

$$P_{X,read} \approx 1 - \prod_{i=1}^{N_{sites}} (1 + [X]/K_{d,X,i})^{-1},$$

where N_{sites} is the number of motif matches for PWM X in the read, $[X]$ is the free concentration of the TF corresponding to the PWM X and $K_{d,X,i} = \exp(-\Delta G_{X,i}/RT)$ is the equilibrium dissociation constant of the binding site i of the TF corresponding to the PWM X . In ref. ⁵⁶, the free concentration of each TF was set to equal the K_d of the consensus sequence. However, for some TFs with a long PWM, exact matches to the consensus sequence are rare, and setting the scoring as described above will result in the occupancy scores 0 for many functional binding sites, reducing the variance of the scores of the variables corresponding to these TFs. To overcome this, we used a normalization approach based on the fact that TFs generally have ~10000-300000 binding sites in the human genome and defined the free concentration of each protein to correspond to the K_d of the strength of its 10000th strongest binding site in the human genome. Thus,

$$P_{X,read} = 1 - \prod_{i=1}^{N_{sites}} (1 + \exp(S_{X,i} - S_{X,10000}))^{-1}$$

where PWM match score of the 10000th strongest match of the PWM X is $S_{X,10000}$ and the PWM match score of the i th site in a read is $S_{X,i}$. This normalization was calculated separately for each PWM and strand. The probability for a pair of TFs ($X+Y$) to occupy a sequence⁵⁶ is

$$P_{XY,read} \approx 1 - \prod_{i=1}^{N_{sites}} \left(\prod_{j=1}^{M_{sites}} (1 + \exp(S_{X,i} + S_{Y,j} - S_{X,10000}))^{-1} \right).$$

Position-specific logistic regression classification

The positional activity scores of the 880 dominating set PWMs and the 7 core promoter PWMs (see **Supplementary Table 12**) were used to train a position-specific logistic regression classifier on the promoter capture STARR-seq data. In contrast to the simple logistic regression described above, the PWM match scores were weighted using the positional activity scores of the corresponding PWM. Thus, instead of occupancy probabilities, scores

$$A_{X,read} = \sum_i a_{X,i} \cdot S_{X,i}$$

were calculated for each sequence and PWM feature X , where $a_{X,i}$ is the positional activity score of PWM X at position i and i runs over all matches of PWM X in a sequence. Regression coefficients were learned separately for both strands for each PWM. Training of the position-specific regression model was done similarly to the training of other logistic regression models. Final model was selected based on area under precision-recall curve on separate validation data. Classification performance on unseen test data is shown for each trained hyperparameter combination in **Supplementary Fig. 4**.

Convolutional neural network classification

The CNN classifiers used the raw fasta sequences as input to learn the features during the training process. The random enhancer CNN and the genomic enhancer CNN were trained using also the reverse complement sequences of the training data, all other models were trained using one orientation only. The CNN models consist of convolutional modules with a 1D convolutional layer followed by batch normalization, ReLu activation and a dropout layer. The number of convolutional modules, the number of convolutional filters per layer, and the dropout rate are hyperparameters that were optimized based on the validation data. The convolutional modules used dilated convolution⁵⁷ so that the dilation rate of the i th layer is i^2 , which allows learning interactions with fewer parameters than fully connected (dense) layers. After the convolutional modules, the final layer is a dense layer of two nodes with sigmoid activation. This CNN architecture was selected by testing it against architectures with varying sized dense layers after the convolutional layers. Adding the dense layers did not improve the performance of the models. The models were built on Keras (<https://keras.io/>; version 2.2.4) using TensorFlow 1.14.0 backend⁵⁸.

Models were trained using the Adam optimizer with default parameter values. Training was stopped once binary accuracy on validation data did not improve within 200 epochs or when the total training time on a single Nvidia Volta V100 GPU exceeded 72 hours (an exception being the “double input” CNN models trained to classify the “binary STARR-seq” data (see below for more details) where training was continued up until 144 hours if needed). Model parameters were initialized using the He uniform variance scaling initializer⁵⁹. All models, except the double input CNN, were trained on batch size 128 as changing the batch

size had a negligible effect on classification performance, and only had one convolutional body and no dense layers (except the final output layer). Batch size for the double input CNN models was determined over grid search between 32 and 64. The other hyperparameters of the CNN models were determined by grid search over the values shown in **Supplementary Table 8**, and the hyperparameters of the final selected models are shown in **Supplementary Table 9**. Classification performance on unseen test data is shown for each trained hyperparameter combination for each trained CNN model in **Supplementary Fig. 4**.

Note on optimal classification of random enhancer STARR-seq data

As seen from **Supplementary Fig. 4**, classifying between random enhancer STARR-seq signal and input sequences is more difficult than for other datasets studied here. We believe that this limited classification accuracy is mainly a feature of the experimental design. Transcription of one molecule of DNA is an inherently probabilistic single-molecule process, and therefore some transcription occurs at random. Some sequences are thus recovered due to this “transcriptional noise”, limiting the maximum classification accuracy. To determine whether a better classification is possible, the replicate experiment of the GP5d random enhancer STARR-seq was utilized to create an unseen test set that contains only those original test set sequences that were also observed in the replicate experiment (2.8% of sequences were observed in both replicates). A balanced number of input library sequences were used as class 0 sequences. Using the final GP5d random enhancer STARR-seq CNN model on this high-confidence test set resulted in ~4% increase in AUprc (**Extended Data Fig. 3b**). The mean(AUprc) $\pm$ std from eight CNN models with different hyperparameters was AUprc_{all}=0.614 $\pm$ 0.004 for the test set with all sequences and AUprc_{common}=0.641 $\pm$ 0.003 for the common sequences between the replicates.

We also further removed the sequences observed more than once in the input library from the 371,390 common STARR-seq sequences between the GP5d random enhancer STARR-seq replicates to evaluate whether possible uneven sequence representation in the input library could affect the classification. For each common STARR-seq library sequence between replicates, the number of matches in the input library was counted by requiring the first 60 bases of the common sequence to match exactly to the first 60 bases of a read 1 sequence in the input library. There were only 6,213 (~0.02%) common sequences between replicates that were observed more than once in the input library (total 999,854,466 input sequences). Removing these 6,213 sequences had a negligible effect on the classification (**Extended Data Fig. 3b**).

As an external evaluation we trained the previously published gapped k-mer SVM classifier^{60,61} on the high-confidence GP5d random enhancer STARR-seq data where the signal set (class 1) sequences were observed in both of the replicates. A balanced set of class 0 sequences were sampled randomly from the input library. We used the default k-mer length (11) and the default decay strength for the center weighted gkm kernel, and tested both “gapped k-mer”, and “gapped k-mer + center weighted” kernels. We optimized the regularization strength over values (1, 0.1, 0.01, 0.001) with the validation data (by calculating area under precision-recall curve). The best hyperparameter combination was obtained by using gapped k-mer kernel and regularization strength=0.1, producing AUprc=0.6207 on the validation data. With the unseen test set (high-confidence sequences) this model obtained AUprc=0.6086. With the full test set from GP5d random enhancer STARR-seq replicate 1 the gapped k-mer SVM obtained AUprc=0.57, which is slightly better than the pairwise logistic regression (0.56) but worse than the CNN (0.62). The gapped k-mer SVM training with the full GP5d random enhancer STARR-seq training set did not complete within approximately three weeks of running time on our computational cluster with 16 parallel threads (gapped k-mer SVM maximum), so performance of a model trained with the full data set could not be evaluated.

Differential expression prediction

A lasso regression model was used to study the extent to which differential gene expression between GP5d and HepG2 cell lines could be predicted using STARR-seq and ATAC-seq derived features. Logarithmic fold-change between GP5d and HepG2 expression values (tpm) was used as the target variable for regression (see section “Chromatin immunoprecipitation (ChIP-seq) and gene expression analysis (RNA-seq and CAGE)” above). The STARR-seq and ATAC-seq peaks were divided into 12 explanatory features based on the cell line in which the peaks were observed and the information about whether the ATAC-seq peaks were promoter proximal (less than 1kB distance to any gene in the target gene set) or not as follows: Common.ATAC.enh.noSTARR; Common.ATAC.enh.yesSTARR; Common.ATAC.prom; Common.STARR.noATAC; GP5d.ATAC.enh.noSTARR; GP5d.ATAC.enh.yesSTARR; GP5d.ATAC.prom; GP5d.STARR.noATAC; HepG2.ATAC.enh.noSTARR; HepG2.ATAC.enh.yesSTARR; HepG2.ATAC.prom; HepG2.STARR.noATAC. Naming “Common.STARR.noATAC” means STARR-seq peaks that were observed in both cell lines and that did not overlap with an ATAC-seq peak, and “HepG2.ATAC.enh.yesSTARR” means HepG2-specific ATAC-seq peaks that are not within 1 kb from target gene TSSs and do overlap with STARR-seq peaks.

For each feature, the logarithmic fold-change at peak summit reported by MACS2 (LFC) was used as the strength of the peak. Peak summit position was used as the position of the feature (ATAC-seq peak summits were used for all other features except the ones that had no overlap with ATAC-seq). Peak score S , meaning the effect of a peak to a TSS that is d bp away from the peak summit for each feature was calculated assuming it decays like:

$$S(\text{peak}) = \text{LFC} \cdot \exp(-c \cdot d / d_{\max}),$$

where LFC is the logarithmic fold-change at peak summit, c is a scaling parameter and $d_{\max}$ is the maximum distance of a peak from the TSS for its effect to be included in the model. This peak score S was used to quantify the effect of each feature in the regression model.

The target genes were split into training (8815 genes), validation (1449 genes) and test (2321 genes) sets according to chromosomes they are in (training: chr1, chr3, chr5, chr7, chr9, chr11, chr13, chr14, chr15, chr16, chr17, chr18, chr19, chr20, chr21, chr22, chrX; validation: chr4, chr6, chr8; test: chr2, chr10, chr11). A lasso regression model was trained using the LassoCV method in scikit-learn⁵⁴ library (version 0.24.1) where the regularization strength (L1 norm) was determined using 5-fold cross-validation during training ($n_{\text{alphas}}=100$, no intercept term). On top of this, the model hyperparameters c and $d_{\max}$ were optimized using the validation data set with a grid search over the values shown in **Supplementary Table 8**. Features were standardized by removing the mean and scaling to unit variance before model training. The best model had the following hyperparameters: $d_{\max}=100,000$ bp; $c=4.3$; regularization strength $\alpha=0.013203848400344683$. This means that the effect of a peak decays to $\frac{1}{2}$ at approximately 16,120 bp from the peak summit. This model obtained $R^2 = 0.1227036350306634$. Regression coefficients of this best performing model are shown in **Supplementary Table 6**.

Interpretation of convolutional neural network classifiers

To test which HT-SELEX PWMs the convolutional neural network had learned, the trained CNN was used as an “oracle” as in ref. ⁶² by embedding 100 sequences drawn randomly from each PWM to random enhancer STARR-seq input library sequences (**Supplementary Table 10**). Each such sequence was embedded at a random position to one of 100 different randomly chosen input sequences (same input sequences used as background for each PWM) and the average enhancer probability over the 100 sequences was calculated for each PWM. When embedding a single PWM per input sequence, first, the position for the embedding was

drawn from random uniform distribution. Next, the embedded sequence was drawn by random from the corresponding PWM. When embedding a motif pair, first the positions of the embedded sequences were drawn at random not allowing overlap between the embedded sequences. Then, both of the embedded sequences were drawn independently from the corresponding PWMs. Thus, both the positions and the distance between the embedded sequences are random. The expected enhancer probability for a sequence with two embedded PWMs given that there are no interactions between them is $p_2 = 1 - (1 - p_1)^2$, where p_i is the enhancer probability of a sequence with i PWMs embedded, representing the cumulative probability for geometric distribution with two trials.

As complementary approaches, we also used *de novo* motif mining as well as DeepLIFT⁸ (version 0.6.12.0) and TF-MoDISco⁹ (version 0.5.14.1) analyses to study the sequence features learned by the random enhancer STARR-seq CNN model. For *de novo* motif mining, we created a set of 10 million 170 bp long DNA sequences that were sampled from random uniform nucleotide distribution. These sequences were then scored with the CNN model, and the top 0.5% of the sequences (50,000) obtaining highest predicted enhancer probabilities were selected for *de novo* motif mining analysis with STREME⁴⁴ (version 5.3.3) using the same parameters as used for mining motifs from random STARR-seq sequences.

DeepLIFT⁸ software shows the importance of each position in a sequence for the final prediction verdict. In our binary classification tasks, positive values correspond to sites that move the prediction verdict towards the active class of sequences and negative values to sites that move the verdict towards the inactive class. We used DeepLIFT to test if the CNN model has learned similar motifs than the simple logistic regression by constructing “CNN activity contribution weight matrices” (CACWM) with the “N-sweep” algorithm using a strategy similar to what was used for generation of activity position weight matrices from the motif library. First, the 20 most important PWM features were selected from the simple logistic regression model (**Supplementary Table 11**) and their consensus sequences obtained. Then, for each position in each of these PWMs, the four possible variants of the sequence were created, and each variant sequence was embedded separately into two different positions in ten randomly sampled background sequences from the random enhancer input library. For each of these 80 sequences per position and per PWM, DeepLIFT contributions were computed against ten dinucleotide-shuffled versions of the sequence itself. The resulting DeepLIFT contribution at position i of the variant was then added to position i of the CACWM for the nucleotide n that is at position i in the variant sequence. Finally, each position of the CACWM was normalized by dividing with the number of different sequences used per variant (20).

To complement the *de novo* motif discovery, we also ran TF-MoDISco⁹ analysis on the same set of top 0.5% highest-scoring sequences according to the random enhancer STARR-seq CNN. First, DeepLIFT contributions were computed for each of the sequences using ten dinucleotide-shuffled references. DeepLIFT hypothetical importance scores were used with TF-Modisco, which was run on default parameters except sliding window size was set to 30, flank size was set to 5, and final minimum cluster size to 60. Motifs discovered by TF-MoDISco were named according to a scan against known motifs with TOMTOM⁴⁵ software (version 5.4.1) with default parameter values. The HNF4 motif in **Extended Data Fig. 4** is named based on manual curation even though that corresponding TF-MoDISco pattern did not have significant (E-value threshold 0.05) matching motifs according to the TOMTOM analysis.

The *TERT* promoter sequences^{13,14,16} were visualized with the DeepLIFT software⁸. The activation values from the wild type and mutated promoter sequences were compared against 15 randomly selected background sequences from the random promoter STARR-seq input and their average signals were visualized. Predicted promoter probabilities of the *TERT* promoters were obtained by scoring the sequences with the CNN trained on promoter capture STARR-seq data.

To visualize pairwise position-specific interactions learned by the CNN classifiers trained on STARR-seq and EPD promoters, respectively, the high-confidence promoter sequences were visualized with the same mutual information (MI) analysis pipeline⁵³ as described above. Here, we generated 10 million sequences of length 120 bp from random uniform nucleotide background and scored each of them with the 10 best (according to binary accuracy on the validation data) STARR-seq promoter capture CNN models and 10 best EPD promoter models. Those sequences obtaining a promoter probability of 0.9 or higher according to each of the 10 models were selected for MI analysis. This resulted in 51,131 sequences for STARR-seq promoter capture CNN and 395,663 sequences for EPD promoter CNN. These numbers together with the MI-plot themselves in **Extended Data Fig. 8f,g** indicate that the STARR-seq data allowed learning models with stricter positional dependencies.

Validation of the predicted promoter mutation effects with external data

To validate the effect of mutations predicted by the CNN model trained on promoter capture STARR-seq data, the model predictions were correlated with a saturation mutagenesis study of the same promoter¹⁵ (see **Extended Data Fig. 8b-d**). The statistical significance of the mutation effects predicted by the CNN model was estimated as follows. First, the predicted promoter probabilities from the CNN model were transformed into log odds scores $\text{logit}(p) = \log(p/(1-p))$, and the predicted effect of each mutation was calculated as the logarithm of odds ratio between the predicted promoter probability of the mutated sequence and the wild type sequence, namely

$$\text{predicted mutation effect} = ME = \text{logit}(p_{\text{mutated}} - p_{\text{WT}}).$$

Then, an empirical one-sided P value was calculated for each predicted *TERT* promoter mutation effect by comparing if the predicted effect in *TERT* promoter is bigger than the predicted effect on shuffled promoter sequences at the same position and for the same type of mutation. This was done by creating 10,000 shuffled sequences (dinucleotide frequencies preserved) of the *TERT* wild type promoter. For each of these, all possible SNPs were introduced, and the predicted mutation effects were calculated for each of them as the logarithm of odds ratio against the predicted promoter probability of the wild type (shuffled) promoter sequence. For each position and mutation type, the empirical P value was calculated as the fraction of the predicted mutation effects on the shuffled sequences that were more extreme than the predicted effect on the wild type *TERT* promoter:

$$p - \text{value} = \frac{1}{N_{\text{pos,mut}}} \sum_i \delta_i(ME > ME_i),$$

for cases where the predicted promoter probability increases due to the mutation and

$$p - \text{value} = \frac{1}{N_{\text{pos,mut}}} \sum_i \delta_i(ME < ME_i),$$

for cases where the predicted promoter probability decreases due to the mutation. $N_{\text{pos,mut}}$ is the total number of shuffled sequences where mutation *mut* was observed at position *pos* and the summation index *i* runs over all such sequences, $\delta_i(ME < ME_i)$ is 1 if $ME < ME_i$ and otherwise 0. All mutations that were non-significant according to either the empirical P value or the P value from the saturation mutagenesis experiment from ref. ¹⁵ were filtered out (threshold P value < 0.05). The Spearman correlations of the model predictions and the saturation mutagenesis logarithmic fold-changes were computed using the “stats.spearmanr” function from SciPy (version 1.1.0, two-sided P value), and are shown in **Extended Data Fig. 8b-d**. The saturation mutagenesis data sets used here are TERT-HEK (HEK293T cells) and TERT-GBM (primary glioblastoma SF7996 cells) from ref. ¹⁵.

Promoter-enhancer interaction analysis using machine learning

The “binary STARR-seq” design allows looking for relatively short-range interactions between promoters and enhancers. To test for any such interactions, we trained several models with a double input CNN architecture, where the promoter and the enhancer parts of the sequence are read in with separate input convolutional bodies (a convolutional body consists of multiple layers of convolutional modules with dilated convolutions) and later connected with a fully connected layer to learn interactions between the outputs of the two convolutional bodies. Depth of the convolutional bodies was optimized with separate validation data along with other hyperparameters detailed in **Supplementary Table 8**. The rationale of this design is that the convolutional modules with dilated convolutions are capable of learning interactions within the promoters and within the enhancers, while the subsequent fully connected layer then integrates information between the promoters and the enhancers.

The search for interactions between promoters and enhancers was implemented by shuffling the training data of the models and keeping the model architecture constant. We trained double input CNN models with four different types of training data: i) The paired readout from the binary STARR-seq experiment with all possible information about interactions between promoters and enhancers intact, referred to as “binary STARR-seq CNN (paired)” in **Fig. 7c**. ii) Permuted training data, where the pairing between the promoter and the enhancer sequences is broken so that any specific interactions between the promoters and enhancers are killed, but all promoters and enhancers come from promoter+enhancer pairs that are active, referred to as “binary STARR-seq CNN (permuted)” in **Fig. 7c**. iii) Training data where only the promoter comes from active promoter+enhancer pairs and the enhancer sequences are sampled randomly from the inactive input pool of enhancer sequences, referred to as “binary STARR-seq CNN (enhancer from input)” in **Fig. 7c**. iv) Training data where only the enhancer comes from active promoter+enhancer pairs and the promoter sequences are sampled randomly from the inactive input pool of promoter sequences, referred to as “binary STARR-seq CNN (promoter from input)” in **Fig. 7c**.

Preprocessing of genomic promoters

Human promoter coordinates were obtained from the eukaryotic promoter database⁶³ (EPD, version 006, hg19) and sequences 100 bp upstream and 20 bp downstream of the TSS were fetched. Promoters overlapping with the extended blacklist described earlier or residing outside of chromosomes 1-22 or X were discarded. The division to training, test and validation sets for machine-learning is detailed in **Supplementary Table 7**. The genomic promoter control set (class 0) was generated by randomly drawing a balanced number of 120 bp sequences (according to the same training, test and validation split) that do not overlap with EPD promoters or regions in the extended blacklist described above. For cancer-associated mutation analysis, the genomic sequence 100 bp upstream and 20 bp downstream of the TSS for *TERT* transcript ENST00000310581.5 was downloaded from Ensembl GRCh37 release 99 Biomart. The top three mutation hotspots in the sequence as well as the recurring mutations (mutations observed in more than one patient) were obtained from ref.¹⁶.

CAGE analysis

The 5' ends of the GP5d CAGE reads contain a 3-bp barcode (ATC) followed by a 6 bp constant sequence (CAGCAG). In addition to removing these, the next 2 bp that were mostly Gs, according to a FastQC quality control report (version 0.11.2; <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), were discarded. The reads were aligned to a combined Phi X 174 + hg19 reference genomes using bwa aln⁶⁴ (version 0.7.10-r789) with parameters ‘-t 8 -B 11 -q 30’. Only the reads mapping to hg19 with MAPQ 30 or higher were extracted and duplicates were removed with samtools rmdup (version 1.3.1). The

strand specific 5'-end coverages of the mapped reads were computed with bedtools⁴¹ genomecov (version 2.27.1). The active promoters were discovered from the mapped CAGE reads by clustering the 5'-ends of the reads with paraclu software⁶⁵(version 9). In total, paraclu called 7365 clusters (peaks) from the GP5d CAGE data fulfilling the following criteria: 1) Cluster is supported by more than 9 unique reads. 2) Cluster cannot be longer than 200 bp. 3) Remove clusters where the maximum 5' end density per base divided by the baseline density is less than 2. 4) Remove any cluster that is contained within a larger cluster. The active GP5d promoters, used as the test set in predicting TSS position, were defined as those EPD test set (see **Supplementary Table 7**) promoters that overlap with a GP5d CAGE peak. Thus, the TSS positions come from EPD.

Supplementary References

- 1 Muerdter, F. et al. Resolving systematic errors in widely used enhancer activity assays in human cells. *Nat Methods* **15**, 141-149 (2018).
- 2 Carninci, P. et al. Genome-wide analysis of mammalian promoter architecture and evolution. *Nat Genet* **38**, 626-635 (2006).
- 3 Wei, B. et al. A protein activity assay to measure global transcription factor activity reveals determinants of chromatin accessibility. *Nat Biotechnol* **36**, 521-529 (2018).
- 4 Lambert, S. A. et al. The Human Transcription Factors. *Cell* **172**, 650-665 (2018).
- 5 Li, K., Chen, Z., Kato, N., Gale, M., Jr. & Lemon, S. M. Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. *J Biol Chem* **280**, 16739-16747 (2005).
- 6 Alipanahi, B., Delong, A., Weirauch, M. T. & Frey, B. J. Predicting the sequence specificities of DNA- and RNA-binding proteins by deep learning. *Nat Biotechnol* **33**, 831-838 (2015).
- 7 Avsec, Z. et al. The Kipoi repository accelerates community exchange and reuse of predictive models for genomics. *Nat Biotechnol* **37**, 592-600 (2019).
- 8 Shrikumar, A., Greenside, P. & Kundaje, A. Learning important features through propagating activation differences. . *Proceedings of the 34th International Conference on Machine Learning* **70**, 3145–3153 (2017).
- 9 Shrikumar, A. et al. Technical Note on Transcription Factor Motif Discovery from Importance Scores (TF-MoDISco) version 0.5.6.5. *arXiv preprint*, doi: arXiv:1811.00416v00415 (2020).
- 10 Yin, Y. et al. Impact of cytosine methylation on DNA binding specificities of human transcription factors. *Science* **356** (2017).
- 11 Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* **9**, R137 (2008).
- 12 Heinz, S., Romanoski, C. E., Benner, C. & Glass, C. K. The selection and function of cell type-specific enhancers. *Nat Rev Mol Cell Biol* **16**, 144-154 (2015).
- 13 Horn, S. et al. TERT promoter mutations in familial and sporadic melanoma. *Science* **339**, 959-961 (2013).
- 14 Huang, F. W. et al. Highly recurrent TERT promoter mutations in human melanoma. *Science* **339**, 957-959 (2013).
- 15 Kircher, M. et al. Saturation mutagenesis of twenty disease-associated regulatory elements at single base-pair resolution. *Nat Commun* **10**, 3583 (2019).
- 16 Zehir, A. et al. Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nat Med* **23**, 703-713 (2017).
- 17 Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**, 357-359 (2012).
- 18 Whyte, W. A. et al. Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* **153**, 307-319 (2013).
- 19 Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol* **34**, 525-527 (2016).
- 20 Pimentel, H., Bray, N. L., Puente, S., Melsted, P. & Pachter, L. Differential analysis of RNA-seq incorporating quantification uncertainty. *Nat Methods* **14**, 687-690 (2017).
- 21 Palin, K. et al. Contribution of allelic imbalance to colorectal cancer. *Nat Commun* **9**, 3664 (2018).
- 22 Buenrostro, J. D., Wu, B., Chang, H. Y. & Greenleaf, W. J. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Curr Protoc Mol Biol* **109**, 21.29.21-21.29.29 (2015).

- 23 Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589-595 (2010).
- 24 Lidschreiber, K. et al. Transcriptionally active enhancers in human cancer cells. *Mol Syst Biol* **17**, e9873 (2021).
- 25 Schwalb, B. et al. TT-seq maps the human transient transcriptome. *Science* **352**, 1225-1228 (2016).
- 26 Zacher, B. et al. Accurate Promoter and Enhancer Identification in 127 ENCODE and Roadmap Epigenomics Cell Types and Tissues by GenoSTAN. *PLoS One* **12**, e0169249 (2017).
- 27 ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57-74 (2012).
- 28 Roadmap Epigenomics Consortium. Integrative analysis of 111 reference human epigenomes. *Nature* **518**, 317-330 (2015).
- 29 Jolma, A. et al. DNA-binding specificities of human transcription factors. *Cell* **152**, 327-339 (2013).
- 30 Nitta, K. R. et al. Conservation of transcription factor binding specificities across 600 million years of bilateria evolution. *Elife* **4**, e04837 (2015).
- 31 Bucher, P. Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. *J Mol Biol* **212**, 563-578 (1990).
- 32 Jin, V. X., Singer, G. A., Agosto-Perez, F. J., Liyanarachchi, S. & Davuluri, R. V. Genome-wide analysis of core promoter elements from conserved human and mouse orthologous pairs. *BMC Bioinformatics* **7**, 114 (2006).
- 33 Grand, R. S. et al. BANP opens chromatin and activates CpG-island-regulated genes. *Nature* (2021).
- 34 Jolma, A. et al. DNA-dependent formation of transcription factor pairs alters their binding specificity. *Nature* **527**, 384-388 (2015).
- 35 Jolma, A. et al. Binding specificities of human RNA-binding proteins toward structured and linear RNA sequences. *Genome Res* **30**, 962-973 (2020).
- 36 Erhard, F. Estimating pseudocounts and fold changes for digital expression measurements. *Bioinformatics* **34**, 4054-4063 (2018).
- 37 Daley, T. & Smith, A. D. Predicting the molecular complexity of sequencing libraries. *Nat Methods* **10**, 325-327 (2013).
- 38 Zorita, E., Cusco, P. & Filion, G. J. Starcode: sequence clustering based on all-pairs search. *Bioinformatics* **31**, 1913-1919 (2015).
- 39 Wunderlich, Z. & Mirny, L. A. Different gene regulation strategies revealed by analysis of binding motifs. *Trends Genet* **25**, 434-440 (2009).
- 40 Li, Q., Brown, J. B., Huang, H. & Bickel, P. J. Measuring reproducibility of high-throughput experiments. *Ann Appl Stat* **5**, 1752-1779 (2011).
- 41 Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841-842 (2010).
- 42 Partridge, E. C. et al. Occupancy maps of 208 chromatin-associated proteins in one human cell type. *Nature* **583**, 720-728 (2020).
- 43 Heinz, S. et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* **38**, 576-589 (2010).
- 44 Bailey, T. L. STREME: Accurate and versatile sequence motif discovery. *Bioinformatics* (2021).
- 45 Gupta, S., Stamatoyannopoulos, J. A., Bailey, T. L. & Noble, W. S. Quantifying similarity between motifs. *Genome Biol* **8**, R24 (2007).

- 46 Cooper, G. M. et al. Distribution and intensity of constraint in mammalian genomic sequence. *Genome Res* **15**, 901-913 (2005).
- 47 Davydov, E. V. et al. Identifying a high fraction of the human genome to be under selective constraint using GERP++. *PLoS Comput Biol* **6**, e1001025 (2010).
- 48 Karolchik, D. et al. The UCSC Table Browser data retrieval tool. *Nucleic Acids Res* **32**, D493-496 (2004).
- 49 Tuupanen, S. et al. Characterization of the colorectal cancer-associated enhancer MYC-335 at 8q24: the role of rs67491583. *Cancer Genet* **205**, 25-33 (2012).
- 50 Lettice, L. A. et al. A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum Mol Genet* **12**, 1725-1735 (2003).
- 51 Gonen, N. et al. Sex reversal following deletion of a single distal enhancer of Sox9. *Science* **360**, 1469-1473 (2018).
- 52 Korhonen, J., Martinmaki, P., Pizzi, C., Rastas, P. & Ukkonen, E. MOODS: fast search for position weight matrix matches in DNA sequences. *Bioinformatics* **25**, 3181-3182 (2009).
- 53 Hartonen, T., Kivioja, T. & Taipale, J. PlotMI: visualization of pairwise interactions and positional preferences learned by a deep learning model from sequence data. *bioRxiv preprint*, doi: <https://doi.org/10.1101/2021.1103.1114.435285> (2021).
- 54 Pedregosa, F. et al. Scikit-learn: Machine Learning in Python. *J Mach Learn Res* **12**, 2825-2830 (2011).
- 55 Hebiri, M. & Lederer, J. How Correlations Influence Lasso Prediction. *IEEE Transactions on Information Theory* **59**, 1846-1854 (2013).
- 56 Granek, J. A. & Clarke, N. D. Explicit equilibrium modeling of transcription-factor binding and gene regulation. *Genome Biol* **6**, R87 (2005).
- 57 Yu, F. & Koltun, V. Multi-scale context aggregation by dilated convolutions. *arXiv preprint*, doi: [arXiv:1511.07122](https://arxiv.org/abs/1511.07122) (2015).
- 58 Abadi, M. et al. TensorFlow: Large-scale machine learning on heterogeneous systems. *arXiv preprint*, doi: [arXiv:1603.04467](https://arxiv.org/abs/1603.04467) (2016).
- 59 He, K., Zhang, Z., Ren, S. & Sun, J. Delving deep into rectifiers: Surpassing human-level performance on imagenet classification. *Proceedings of the IEEE international conference on computer vision.*, doi: [10.1109/ICCV.2015.1123](https://doi.org/10.1109/ICCV.2015.1123) (2015).
- 60 Ghandi, M., Lee, D., Mohammad-Noori, M. & Beer, M. A. Enhanced regulatory sequence prediction using gapped k-mer features. *PLoS Comput Biol* **10**, e1003711 (2014).
- 61 Lee, D. LS-GKM: a new gkm-SVM for large-scale datasets. *Bioinformatics* **32**, 2196-2198 (2016).
- 62 Avsec, Z. et al. Base-resolution models of transcription-factor binding reveal soft motif syntax. *Nat Genet* **53**, 354-366 (2021).
- 63 Dreos, R., Ambrosini, G., Groux, R., Cavin Perier, R. & Bucher, P. The eukaryotic promoter database in its 30th year: focus on non-vertebrate organisms. *Nucleic Acids Res* **45**, D51-D55 (2017).
- 64 Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754-1760 (2009).
- 65 Frith, M. C. et al. A code for transcription initiation in mammalian genomes. *Genome Res* **18**, 1-12 (2008).

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☐ ☒ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☐ ☒ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection No specialized software was used for data acquisition.

Data analysis

The following previously described software were used in the data analysis and are described and cited in the Methods: ChIP-seq reads were aligned to hg19 genome with bowtie2 software (version 2.2.4). All peak calling was done with MACS2 (version 2.1.1) software. Bedtools (versions 2.25.0 and 2.29.2 when using -wao option) was used for genome arithmetics. Super-enhancers were calculated using the ROSE pipeline (version 0.1). ATAC-seq fastq files were processed using an in-house pipeline comprising of following pieces of software: TrimGalore (version 0.4.3), BWA aligner (version 0.7.15), Picard (version 2.9.2) and broad-peak calling by MACS2 (version 2.1.1). Fold change estimates for count data were calculated using the PsilFC function in R package lfc (version 0.2.1). Random enhancer STARR-seq paired end reads were combined using FLASH program version 1.2.11. For library complexity estimation, random STARR-seq library sequences were first clustered using Starcode program version 1.3. Both genomic and random STARR-seq library complexities were estimated using Preseq program version 2.0.0. Genomic STARR-seq reads were aligned to hg19 genome using bowtie2 (version 2.2.4) and peaks called with MACS2 (version 2.1.1) and IDR (version 2.0.3) programs. De novo motif mining for ATAC-seq and ChIP-seq peaks and functional enhancers was performed with HOMER software (version 4.10.3). From the sequences enriched by the STARR-seq experiments from random enhancer, random promoter random enhancer data and ATI assay and specific positions in the TSS-aligned random promoter STARR-seq sequences the de novo motif mining was done using the Autoseed program (as in Nitta et al, Elife 4, e04837, 2015). Additional de novo motif mining from random enhancer STARR-seq and random sequences scored by the random enhancer STARR-seq CNN model was conducted using STREME program (version 5.3.3). STARR-seq derived motifs were compared to known TF motifs using TOMTOM (versions 5.0.5 and 5.3.3 used for motif and random library, respectively). Human genome (hg19) coordinates of the SOX9 enhancer were calculated with the USCS genome LiftOver tool from mouse mm9 coordinates (from <http://genome.ucsc.edu/cgi-bin/hgLiftOver>). Random promoter-enhancer pairs were filtered for adapter sequences using cutadapt software version 1.9.1 and sequences mapping to the plasmid backbone according to bowtie2 were removed. Cutadapt was also used in mapping the TSS position based on template switching. PWM match scores to DNA were calculated using MOODS software (version 1.9.3). Logistic regression classifiers were implemented using the LogisticRegression function from scikit-learn library (version 0.21.3). Lasso regression models were implemented using LassoCV method in scikit-learn (version 0.24.1). Convolutional neural network (CNN)

classifiers were implemented using Keras (version 2.2.4-tf) with TensorFlow 1.14.0 backend. BWA aligner version 0.7.15-r1142-dirty was used in creating the extended blacklist for genomic machine learning analyses. DeepLift (version 0.6.12) was used to visualize the features the promoter STARR-seq trained CNN used for predicting promoter activity. Motif discovery from the random enhancer STARR-seq CNN model was conducted using TF-MoDISco program (version 0.5.14.1). TOMTOM version 5.4.1 was used to compare TF binding motifs learned by the CNN to de novo motifs from random enhancer STARR-seq sequences. FastQC software (version 0.11.2) was used for CAGE data quality control. CAGE reads were aligned to combined Phi X 174 and hg19 genomes with the BWA aligner (version 0.7.10-r789) and the CAGE mapped read clustering was done using paraclu software version 9. RNA-seq differential expression analysis was conducted using kallisto and sleuth program versions 0.46.1 and 0.30.0, respectively. Gene set enrichment analysis was done using preranked analysis with GSEA (version 4.1.0). SciPy (version 1.1.0) functions `stats.ttest_rel` and `stats.spearmanr` were used to compute paired Student's t-test and Spearman correlations, respectively.

All custom code central to the study used in the data analysis is described in the Methods and available at Zenodo with accession 10.5281/zenodo.5159644 as mentioned in the Code Availability Statement. The code is divided into six packages: 1) StarrTrack: Java code for processing motif STARR-seq library data; 2) AssignTSS: Perl script that assigns the TSS positions based on the template-switch data; 3) CountMotifPairs: Two R scripts that count motif match pairs from promoter enhancer pairs and motif match spacings and orientations from enhancers, respectively; 4) trainCNN: Python scripts for training the CNN classifiers; 5) trainLogReg: Python scripts for training the logistic regression classifiers; 6) trainReg: Python scripts for training the differential expression predictor.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All sequence data generated in this study is available under GEO accession GSE180158. All pre-trained machine learning models are available at Zenodo with accession 10.5281/zenodo.5101420. Training, test, and validation data sets for the CNN models are available at Zenodo with accession 10.5281/zenodo.5101420. Genome browser session is available at UCSC portal with tracks for all genomic data sets generated in this study (https://genome.ucsc.edu/s/kivioja/Sahu_et_al_Human_regulatory_elements).

ENCODE blacklisted genomic regions for hg19 (accession ENCSR636HFF) were downloaded from ENCODE, RepeatMasker file for hg19 was downloaded using the UCSC table browser. The Eukaryotic Promoter Database (EPD) for human TSSs can be found from: https://epd.epfl.ch/EPDnew_database.php. In addition, transcript annotations downloaded from Ensembl (GRCh37, release 101) were used. The saturation mutagenesis results of the TERT promoter can be found from: <https://doi.org/10.17605/OSF.IO/75B2M>. GERP conservation scores for the hg19 reference genome can be found from: <http://mendel.stanford.edu/SidowLab/downloads/gerp/>. The following datasets were downloaded from the ENCODE portal: ATAC-seq (ENCSR042AWH, replicate 1), histone modification ChIP-seq experiments for H3K27ac (ENCSR000AMO), H3K27me3 (ENCSR000AOL), H3K9me3 (ENCSR000ATD), and H3K4me1 (ENCCFF424GUI), as well as ChIP-seq data sets for TP53 (ENCSR980EGJ), MED1 (ENCCFF493UFO), and MED13 (ENCCFF003HBS). ChIP-seq peak sets were also downloaded from GEO accession GSE104247. Super-enhancers for HepG2 were downloaded from <http://www.licpathway.net/sedb>. Previously published RNA-seq data used in the study is available at EGA (accession <https://ega-archive.org/studies/EGAS00001002966>).

Accession numbers for all data sets used in this study are also mentioned in Data Availability Statement.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

The achievable sample sizes in STARR-seq experiments are limited by cloning, cell culture and other steps of the large-scale experiments. The ultra-high complexities of the input libraries were verified using state-of-art computational tools as described in Methods. The reliability of the biological conclusions made from the achievable sample sizes was ensured by i) manual inspection of the genomic peaks, ii) replicate comparisons, iii) statistical testing, and iv) power analysis where appropriate. The numbers of the sequencing reads used in each analysis are listed in Supplementary Tables.

Data exclusions

In all experiments, sequencing reads were filtered using defined quality parameters and the genomic reads overlapping with the ENCODE blacklisted regions were excluded. IRF3 ChIP-seq signals were weak in HepG2 cells based on the lack of peak overlaps with motif sites and the manual inspection of the top peaks, and they were excluded from the downstream analysis.

Replication

STARR-seq experiments were performed in two replicates with random enhancer libraries in GP5d and HepG2 cells and with random promoter-enhancer libraries in GP5d cells; genomic STARR-seq experiments were performed in two replicates in HepG2 cells and in four different conditions in GP5d cells (wild type and TP53-null GP5d cells using both methylated and non-methylated input DNA libraries); experiments with random promoter-enhancer libraries in HepG2 and RPE1 cells had no replicates. RNA-seq experiments were performed in three replicates, ChIP-seq and ATAC-seq from one sample per condition.

Similar conclusions were obtained from independently generated data sets and the replicate experiments showed high concordance (Extended Data Figures 2a and 5a).

Randomization

Not applicable. Experiments were performed on uniform biological material i.e. commercial cell lines, so randomization for different experimental groups was not relevant for this study. Randomization of the sequencing reads for the purposes of specific statistical or computational analysis, such as for training, test, and validation sets used in the machine learning analyses, is described for each specific analysis in the Methods section.

Blinding

Analyses were performed using computational algorithms for large data sets of sequencing reads, and thus blinding of the investigators was not relevant for this study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

- n/a Involved in the study
- ☐ ☒ Antibodies
- ☐ ☒ Eukaryotic cell lines
- ☒ ☐ Palaeontology and archaeology
- ☒ ☐ Animals and other organisms
- ☒ ☐ Human research participants
- ☒ ☐ Clinical data
- ☒ ☐ Dual use research of concern

Methods

- n/a Involved in the study
- ☐ ☒ ChIP-seq
- ☒ ☐ Flow cytometry
- ☒ ☐ MRI-based neuroimaging

Antibodies

Antibodies used

H3K27ac, H3K9me3, and H3K27me3 (C15410196, C15410193, and C15410195, Diagenode, respectively); FOXA1 (#ab23738, Abcam); p53, HNF4a, IRF3 and CTCF [sc-126x (DO-1), sc-8987x (H-171), sc-33641x (SL-12), and sc-15914x (C-20), Santa Cruz, respectively]; SMC1 (A300-055A, Bethyl lab), and normal mouse, rabbit and goat IgG from Santa Cruz (#sc-2025, #sc-2027 and #sc-2028, respectively)

Validation

The anti-H3K27ac polyclonal antibody is raised in rabbit against the region of histone H3 containing the acetylated lysine 27 (H3K27ac), using a KLH-conjugated synthetic peptide. It is recommended for detecting H3K27ac in ChIP experiments in human by the manufacturer, and there is validation data and >50 citations available for this antibody on the manufacturer's website (<https://www.diagenode.com/en/p/h3k27ac-polyclonal-antibody-premium-50-mg-18-ml>).

The anti-H3K9me3 polyclonal antibody is raised in rabbit against the region of histone H3 containing the trimethylated lysine 9 (H3K9me3), using a KLH-conjugated synthetic peptide. It is recommended for detecting H3K9me3 in ChIP experiments in human by the manufacturer, and there is validation data and >30 citations available for this antibody on the manufacturer's website (<https://www.diagenode.com/en/p/h3k9me3-polyclonal-antibody-premium-50-mg>).

The anti-H3K27me3 polyclonal antibody is raised in rabbit against the region of histone H3 containing the trimethylated lysine 27 (H3K27me3), using a KLH-conjugated synthetic peptide. It is recommended for detecting H3K27me3 in ChIP experiments in human by the manufacturer, and there is validation data and >60 citations available for this antibody on the manufacturer's website (<https://www.diagenode.com/en/p/h3k27me3-polyclonal-antibody-premium-50-mg-27-ml>).

The anti-FOXA1 polyclonal antibody is raised in rabbit against the synthetic peptide within human FOXA1 aa 450 to the C-terminus (C terminal) conjugated to keyhole limpet haemocyanin. It is recommended for detecting FOXA1 in ChIP experiments in human by the manufacturer, and there is validation data and >100 citations available for this antibody on the manufacturer's website (<https://www.abcam.com/foxa1-antibody-chip-grade-ab23738.html?productWallTab=ShowAll>).

The anti-p53 (DO-1) is a mouse monoclonal antibody raised against a short amino acid sequence containing Ser315 phosphorylated p53 of human origin. It is a ChIP-grade antibody recommended for detecting human p53 by the manufacturer. There is validation data on the manufacturer's website, including 5780 citations for previous literature (<https://www.scbt.com/p/p53-antibody-do-1>).

The anti-IRF3 (SL-12) is a mouse monoclonal antibody raised against recombinant IRF-3 fusion protein corresponding to human IRF-3 (amino acids 56-427). It is a ChIP-grade antibody recommended for detecting human IRF3 by the manufacturer. There is validation data on the manufacturer's website, including 46 citations for previous literature (<https://www.scbt.com/p/irf-3-antibody-sl-12>).

The anti-HNF4a (H171) is a rabbit polyclonal antibody raised against an epitope corresponding to amino acids 295-465 mapping at the C-terminus of HNF-4α of human origin. It is a ChIP-grade antibody recommended for detecting human HNF4a by the manufacturer. There is validation data on the manufacturer's website, including 71 citations for previous literature (<https://www.scbt.com/p/hnf-4alpha-antibody-h-171>).

The anti-CTCF (C-20) is a goat polyclonal antibody raised against an epitope mapping near the C-terminus of CTCF of human origin. It is a ChIP-grade antibody recommended for detecting human CTCF by the manufacturer with >20 citations available for this antibody (<https://www.citeab.com/antibodies/782679-sc-15914-ctcf-antibody-c-20>).

The anti-SMC1 is a rabbit polyclonal antibody raised against an epitope mapping between 1175 and C-terminus of SMC1 of human origin. It is recommended for detecting human SMC1 by the manufacturer with >180 citations available for this antibody (<https://www.bethyl.com/product/A300-055A/SMC1+Antibody>).

Normal IgGs from Santa Cruz are commonly used controls.

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	Colon cancer cell line GP5d (Sigma #95090715), liver cancer cell line HepG2 (ATCC #HB-8065), retinal pigmented epithelial cell line hTERT-RPE1 (ATCC #CRL-4000).
Authentication	All cell lines were directly obtained from trusted vendors (ATCC, Sigma) and not from other laboratories, and only low-passage cells were used in the experiments. Cell lines were not authenticated.
Mycoplasma contamination	All cell lines tested negative for mycoplasma contamination upon purchase and were routinely monitored as per standard good laboratory practices.
Commonly misidentified lines (See ICLAC register)	Cell lines used in this study are not on the list of commonly misidentified cell lines

ChIP-seq

Data deposition

- ☒ Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
- ☒ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

May remain private before publication.

Raw and processed reads are available under GEO accession GSE180158.

Files in database submission

H3K27ac_Mock_HepG2_ChIP_10_S10_L1-2.fastq.gz
 p53_Mock_HepG2_ChIP_7_S7_L1-2.fastq.gz
 IRF3_Mock_HepG2_ChIP_13_S13_L1-2.fastq.gz
 H3K27ac_SS-NLE_HepG2_ChIP_11_S11_L1-2.fastq.gz
 p53_SS-NLE_HepG2_ChIP_8_S8_L1-2.fastq.gz
 IRF3_SS-NLE_HepG2_ChIP_14_S14_L1-2.fastq.gz
 H3K27ac_5-FU_HepG2_ChIP_12_S12_L1-2.fastq.gz
 p53_5-FU_HepG2_ChIP_9_S9_L1-2.fastq.gz
 IRF3_5-FU_HepG2_ChIP_15_S15_L1-2.fastq.gz
 rlg_Mock_HepG2_ChIP_1_S1_L1-2.fastq.gz
 rlg_SS-NLE_HepG2_ChIP_2_S2_L1-2.fastq.gz
 rlg_5-FU_HepG2_ChIP_3_S3_L1-2.fastq.gz
 mlg_Mock_HepG2_ChIP_4_S4_L1-2.fastq.gz
 mlg_SS-NLE_HepG2_ChIP_5_S5_L1-2.fastq.gz
 mlg_5-FU_HepG2_ChIP_6_S6_L1-2.fastq.gz
 Input_HepG2_ChIP_21_S21_L1-2.fastq.gz
 GP5d-Ctrl-p53-DO-1_S2_R1_001.fastq.gz
 GP5d-5-FU_p53-DO-1_S3_R1_001.fastq.gz
 GP5d-5-FU_mlg_S1_R1_001.fastq.gz
 CTCF2_GP5d_S11_R1_001.fastq.gz
 glgG-GP5D_S1_R1_001.fastq.gz
 GP5D-H3K27ac_S45_R1_001.fastq.gz
 GP5D-rlgG_S39_R1_001.fastq.gz
 SMC1-GP5D_S8_R1_001.fastq.gz
 GP5D-H3K9me3_S63_R1_001.fastq.gz
 rlgG-GP5D_S2_R1_001.fastq.gz
 FOXA1_2_GP5d_S12_R1_001.fastq.gz
 HNF4a2_GP5d_S13_R1_001.fastq.gz
 rlgG2_GP5D_S8_R1_001.fastq.gz
 mlgG-GP5D_S3_R1_001.fastq.gz
 GP5d-1_H3K27me3_S10_R1_001.fastq.gz
 GP5d-rlgG-hm_S8_R1_001.fastq.gz
 H3K27ac_Mock_HepG2_vs_Input_peaks.narrowPeak
 p53-Mock_HepG2_vs_Input_peaks.narrowPeak
 IRF3-Mock_HepG2_vs_Input_peaks.narrowPeak
 H3K27ac_SS-NLE_HepG2_vs_Input_peaks.narrowPeak
 p53-SS-NLE_HepG2_vs_Input_peaks.narrowPeak
 IRF3-SS-NLE_HepG2_vs_Input_peaks.narrowPeak
 H3K27ac_5-FU_HepG2_vs_Input_peaks.narrowPeak
 p53-5-FU_HepG2_vs_Input_peaks.narrowPeak
 IRF3-5-FU_HepG2_vs_Input_peaks.narrowPeak
 Ctrl_TP53_vs_mlgG_peaks.narrowPeak
 5FU_TP53_vs_mlgG_peaks.narrowPeak
 CTCF2_GP5D_vs_glgG2_peaks.narrowPeak
 H3K27ac_vs_rlgG_GP5d_peaks.narrowPeak
 SMC1-GP5D_vs_rlgG_peaks.narrowPeak
 H3K9me3_GP5D_broad_vs_rlgG_peaks.broadPeak

FOXA1_2_GP5D_vs_rlgG2_peaks.narrowPeak
 HNF4a_2_GP5D_vs_rlgG2_peaks.narrowPeak
 GP5d-1_H3K27me3_vs_rlgG-hm_peaks.broadPeak

Genome browser session
 (e.g. [UCSC](https://genome.ucsc.edu))

https://genome.ucsc.edu/s/kivioja/Sahu_et_al_Human_regulatory_elements

Methodology

Replicates

The main findings of the study are based on the STARR-seq experiments, and ChIP-seq data for was used to support the conclusions. Due to the large number of different ChIP-seq data sets needed, we only used one replicate for each condition.

Sequencing depth

Mapped reads for GP5d-TP53: total = 63021885; unique = 44989603
 Mapped reads for GP5d-rlgG control for TP53: total = 40330794; unique = 28699379
 Mapped reads for GP5d-FOXA1: total = 47301290; unique = 4007819
 Mapped reads for GP5d-rlgG control for FOXA1: total = 49338820; unique = 43678360
 Mapped reads for GP5d-CTCF: total = 32829873; unique = 28644653
 Mapped reads for GP5d-rlgG control for CTCF: total = 26247818; unique = 23128132
 Mapped reads for GP5d-H3K27ac: total = 61776858; unique = 50807468
 Mapped reads for GP5d-rlgG control for H3K27ac: total = 37885351; unique = 32031305
 Mapped reads for GP5d-H3K9me3: total = 20163128; unique = 17797650
 Mapped reads for GP5d-rlgG control for H3K9me3: total = 49338820; unique = 43678360
 Mapped reads for GP5d-H3K27me3: total = 35415410; unique = 24816970
 Mapped reads for GP5d-rlgG control for H3K27me3: total = 31003987; unique = 22463136
 Mapped reads for GP5d-HNF4a: total = 20180294; unique = 15124792
 Mapped reads for GP5d-rlgG control for HNF4a: total = 48864528; unique = 30269040
 Mapped reads for GP5d-SMC1: total = 41817385; unique = 36074245
 Mapped reads for GP5d-rlgG control for SMC1: total = 49338820; unique = 43678360
 Mapped reads for HepG2-p53-SS-NLE_HepG2_vs_Input : total = 23008852; unique = 18888219
 Mapped reads for HepG2-Input control for p53-SS-NLE_HepG2: total = 30690201; unique = 26599619
 Mapped reads for HepG2-p53-Mock_HepG2_vs_Input : total = 24501270; unique = 19517558
 Mapped reads for HepG2-Input control for p53-Mock_HepG2: total = 30690201; unique = 26599619
 Mapped reads for HepG2-p53-5-FU_HepG2_vs_Input : total = 16674402; unique = 10798674
 Mapped reads for HepG2-Input control for p53-5-FU_HepG2: total = 30690201; unique = 26599619
 Mapped reads for HepG2-IRF3-SS-NLE_HepG2_vs_Input : total = 16165814; unique = 7363592
 Mapped reads for HepG2-Input control for IRF3-SS-NLE_HepG2: total = 30690201; unique = 26599619
 Mapped reads for HepG2-IRF3-Mock_HepG2_vs_Input: total = 11264345; unique = 5765858
 Mapped reads for HepG2-Input control for IRF3-Mock_HepG2: total = 30690201; unique = 26599619
 Mapped reads for HepG2-IRF3-5-FU_HepG2_vs_Input: total = 19588013; unique = 9655159
 Mapped reads for HepG2-Input control for IRF3-5-FU_HepG2 total = 30690201; unique = 26599619
 Mapped reads for HepG2-H3K27ac_SS-NLE_HepG2_vs_Input: total = 30963011; unique = 22680118
 Mapped reads for HepG2-Input control for H3K27ac_SS-NLE_HepG2 total = 30690201; unique = 26599619
 Mapped reads for HepG2-H3K27ac_Mock_HepG2_vs_Input: total = 40104064; unique = 28382584
 Mapped reads for HepG2-Input control for H3K27ac_Mock_HepG2 total = 30690201; unique = 26599619
 Mapped reads for HepG2-H3K27ac_5-FU_HepG2_vs_Input: total = 31156671; unique = 21425466
 Mapped reads for HepG2-Input control for H3K27ac_5-FU_HepG2 total = 30690201; unique = 26599619

Antibodies

H3K27ac, H3K9me3, and H3K27me3 (C15410196, C15410193, and C15410195, Diagenode, respectively); FOXA1 (#ab23738, Abcam); p53, HNF4a, IRF3 and CTCF (sc-126x, sc-8987x, sc-33641x, and sc-15914x, Santa Cruz, respectively); SMC1 (A300-055A, Bethyl lab), and normal mouse, rabbit and goat IgG from Santa Cruz (#sc-2025, #sc-2027 and #sc-2028, respectively)

Peak calling parameters

Peak calling was performed using MACS2 with default parameters (narrow peaks were called for all samples except broad peaks for repressive histone modifications).

Data quality

Correct TF motifs were discovered from the ChIP-seq peaks for all samples except IRF3 ChIP-seq in HepG2, and thus IRF3 data was not used in further analysis.

Software

Bowtie2 (Langmead, & Salzberg, Nat Methods 9, 357-359, 2012)
 MACS2 (Zhang et al., Genome Biol. 9, pp. R137, 2008)

Peer Review Information

Journal: Nature Genetics

Manuscript Title: Sequence determinants of human gene regulatory elements

Corresponding author name(s): Professor Jussi Taipale

Reviewer Comments & Decisions:

Decision Letter, initial version:
--

27th May 2021

Dear Jussi,

Your Article, "Sequence determinants of human gene regulatory elements" has now been seen by 3 referees. I apologize for the long review process. Unfortunately, reviewer #4 has not submitted a timely report despite our multiple chase emails. We have now decided to proceed based on the current reviews.

You will see from the reviewers' comments copied below that while they find your work of considerable potential interest, they have raised quite substantial concerns that must be carefully and thoroughly addressed. In light of these comments, we cannot accept the manuscript for publication, but would be interested in considering a revised version that addresses these serious concerns.

Reviewer #1 thinks that the dataset is valuable and that the findings are potentially exciting but that the manuscript is very complex. The reviewer feels that it should either be split into several studies or that you need to restructure it; we recommend the latter. Importantly, the reviewer asks for all the data/methods to be adequately provided/explained. They raise some salient technical issues but these are not necessarily damaging.

Reviewer #2 is positive about this study technically and thinks that it is thought-provoking. They have

a number of very insightful and constructive comments; some are about data interpretation/display, others about additional analyses.

Reviewer #3's comments are not very detailed, unfortunately. The reviewer thinks that the manuscript is "impenetrable", which is reminiscent of what Reviewer #1 said. They note that the claims are potentially important but seem unsubstantiated at this stage; the reviewer doesn't provide details, though.

We hope you will find the referees' comments useful as you decide how to proceed. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

If you choose to revise your manuscript taking into account all reviewer and editor comments, please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact me if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If revising your manuscript:

*1) Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available http://www.nature.com/ng/authors/article_types/index.html here. Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary: <https://www.nature.com/documents/nr-reporting-summary.pdf>
It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.
A revised checklist is essential for re-review of the paper.

Please be aware of our <https://www.nature.com/nature-research/editorial-policies/image-integrity> guidelines on digital image standards.

You may use the link below to submit your revised manuscript and related files:

[REDACTED]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If

you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Genetics or published elsewhere. Should your manuscript be substantially delayed without notifying us in advance and your article is eventually published, the received date would be that of the revised, not the original, version.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Nature Genetics is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit www.springernature.com/orcid.

Thank you for the opportunity to review your work.

Sincerely,

Tiago

Tiago Faial, PhD
Senior Editor
Nature Genetics
<https://orcid.org/0000-0003-0864-1200>

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

In their manuscript "Sequence determinants of human gene regulatory elements" Biswajyoti Sahu et al. performed a number of very interesting reporter assays (STARR-Seq) and in vitro binding (ATI) experiments, as well as created a number of supporting RNA-seq, ATAC-seq and ChIP-seq data sets. The result is a very interesting (but also very complex) read out of regulatory elements in (mostly) two cancer cell-lines (HepG2 and GP5d). There are multiple different STARR-Seq assays in this study and the authors branch out into studying effects of methylation, the interaction of regulatory sequences ("promoter"- "enhancer" interactions) as well as the effects of a specific TF knockout (TP53). This all is supplemented by an in-depth modeling analysis using regression, gapped-kmer or CNN models.

Trying to put all that into a single study comes at the cost of oversimplifying a story in the main text

(without a comprehensive discussion of individual limitations) while providing a very long method description that is too complex to follow for anyone who is not acquainted with the study. My first suggestion would be to "divide and conquer" and to publish this work as a set of two to three articles in the "same issue" of a journal. I believe that we are looking at some very interesting work here, which is really not well represented the way it is currently written up. If splitting up is no option, I would highly recommend to write a structured supplemental note where the individual experiments are motivated and outlined, and most importantly their limitations discussed.

Another high-level criticism is the availability of models and raw as well as processed data. While the authors announce that models and raw data will be released, this is something that should be done before submitting revisions of the manuscript. The reviewers need to see that all data was deposited with the appropriate meta-information. Specifically, all data should be deposited through NCBI GEO to allow that also processed data is released. Similarly, data/methods cannot be referenced with a manuscript "in preparation" (L733). Either this manuscript is available as pre-print or the information is provided as "personal communication", in which case a sufficient description has to be provided to the reader/reviewers.

As briefly mentioned above, the current version of the main manuscript appears to me more of a big picture story telling; while many important details are only available in the methods and a discussion of experimental limitations is missing. In this context it seems most important that even if trying to simplify for a broad readership, a correct picture is conveyed. For me issues arise for the following topics:

(1) It is essential to mention the applied experimental methods early on (i.e. in the abstract) and the authors should refrain from comparing to an "aggregated length" of tested sequences throughout the manuscript. The authors tested millions of short sequences, they never tested long sequences and a concatenated length is of no relevance here as these sequences did not act in that aggregate form.

(2) The authors talk about "evolved" sequences, but what they mean is that they tested random sequences and that a proportion of those showed activity. This has nothing to do with molecular evolution. In some contexts, it might be sufficient to talk about motifs/sequences that are enriched/overrepresented among those activating expression.

(3a) The authors are doing an unusual normalization of STARR-Seq data, which is typically used as signal over background measure. Due to the high library complexity, sequencing the genomic background is uneconomical (L846ff) and the authors try to normalize differently (e.g. by comparing motif enrichments vs a random sample of the input). This however cannot correct for artifacts in the STARR-Seq data or for more subtle effects like GC content. The authors need to discuss these technical aspects of their data (and not just in the methods).

(3b) STARR-Seq (like also MPRA/CRE-seq approaches) seem inherently biased (e.g. due to a minimal promoter) towards activating rather than repressing effects. How does this impact your study and the drawn conclusions?

(3c) STARR-Seq assays also cause a strong cellular "immune" response and you seem to be dealing with a rather high number of plasmids per cell (L876), how does that impact your results? What about plasmid silencing?

(Very incomplete list of) minor/other comments:

- L67: provide length/context of TF binding motif MPRA
- L110: active TF identification (ATI) need more of an introduction
- L220: discuss how this relates to "primed" enhancers and whether these are active enhancers in other cell-types that only become active because they are moved into an active chromatin context.
- L347: "Molecular evolution approach" -- Is there a prior definition of what this entails? I am having issues with the term evolution for this study.
- L401ff: The statement about TFs as basic unit makes sense and is probably not challenged. The argument whether "regulatory elements are not atomic units of gene expression" seems to be semantic one, especially as the authors argue for an additive effect of multiple TFs with some activation limit. Considering that the authors are testing these TFs in (very) close proximity (due to technical limitations), it seems an unsupported statement that there would be no length-restricted local neighborhood within the TFs act additively on expression regulation and that these should not be called regulatory elements.
- L522ff: Details about amount of DNA and cycles missing
- L539ff: Amount of material, estimate of oligo complexity
- L558ff: It remains unclear to me how the reporter gene is inserted in between "promoter" and "enhancer" random oligo regions, the size of 555 bp seems too short to include it. Please clarify.
- L647ff: Template switching seems to be the required method for reading the "promoter"- "enhancer" STARR-seq library. This needs to be clearer. Also Fig4a (L1890) needs a complete description of the process. You should also bring the corresponding analysis methods closer in text (i.e. L995ff and L1010ff).
- L729: Description of bioinformatics analysis of data as well as of enriched motifs/known factors seems to be missing.
- L830ff: In a previous analysis you used the LFC package, why are you not using it here?
- L1000: "Hamming distance of less than 3". You are probably trying to handle synthesis and sequencing errors here. Please provide more insight into why you are doing things and why you are picking these thresholds.
- L1070: You are using Ensembl (L) and EPD (L1031) for TSS annotation across different analyses, you therefore need to specify the source in each case.
- L1098: Why adding a pseudo count of 10?
- L1150ff: Regularization does not protect from issues with correlated features. It might slightly help the interpretation, but it does not solve the actual issue.

- L1843: Not STARR-seq on its own, but the combination of readouts reveals the different types of regulatory elements. Especially as STARR-Seq does not show signal for some otherwise defined elements.

Textual errors:

- L187: [the] GP5d genome
- L702: correct sentence around "lysed"
- L867: which -> with
- L1817: delete "relatively"

Overall, this is a great set of experiments and a very exciting resource. I hope the authors can make all this a bit better accessible to the reader. Even as a very interested reader, I could only get through a very thin layer of this work.

Reviewer #2:

Remarks to the Author:

The authors present an interesting study examining the transcriptional activity of 400 billion DNA bases in three cellular contexts using STARR-seq. Collectively, their study examines ~100 times the sequence capacity of the human genome, and therefore serves as a strong platform for a large-scale unbiased view of the sequence determinants of human gene regulatory element activity.

Several key observations are presented based on the data:

1. There are three classes of enhancers, with different mechanisms and motif content
2. Transcription factors (TFs) generally have (weak) additive effects
3. Only a few TFs are strongly active in a cell type, with the strongest differences between cells representing known TFs that are important for specification and/or function of the specific lineages
4. Only a subset of TFs have TSS positional dependency
5. There is no evidence for 'beyond additive' interactions between promoters and enhancers

Overall this is a well designed and well executed study. I expect it will be very interesting for the community and will spark a good amount of discussion and debate. That said, I have several comments, most of which involve data interpretation.

Analysis/presentation/interpretation issues:

1. "The most active motifs displayed similar activities when placed in different sequence contexts, and between experiments using two different basal promoters, δ 1-crystallin and CpG-free EF1 α promoters" – not sure I totally agree with that 2nd part. For the top motifs (p53, IRF HT2 in Ext Data Fig 2B), it looks like 1000-fold vs 30-fold induction. I agree that the rankings look similar, but I would hardly call that "similar activity". I get that EF1 α is a stronger promoter, but the wording should be tweaked a bit here.
2. How was a single TF chosen to plot the expression levels shown in Figure 1C and Figure 1D? any of those ETS motifs could correspond to several different Ets-family proteins, for example (same with NFAT/Fox/IRF/others). Something like a violin plot, which would show all possible TFs that recognize each motif, might be more informative.
3. "As the library contained each single-base substitution to the p53 family consensus sequence, we

were able to generate an activity position weight matrix (PWM) for the consensus. The activity PWM was highly similar to the SELEX derived motif for the p53 family” – can a similar exercise be performed for some other TFs? P53 has a very information-rich motif (and very high activity), so I would think it might be a bit of an outlier in terms of TF behavior in these assays.

4. Only five de novo motifs are shown in Extended Data Fig. 5a. One of these looks like a likely ‘composite element’. This is presented as evidence that “the backbone of the transcriptional system is based on individual TFs acting together without strict spacing preferences or grammar”. I would need to see at least the top 40 de novo motifs in the random enhancer library, along with their p-values, predicted frequencies of occurrence, and enrichment scores to really buy this argument. This would also help bolster the results of the regression analysis.

5. What happens if you use the de novo motifs in the regression analysis? I like seeing the results that use known motifs (since it is less circular), but it would be interesting to see how that compares in overall performance, and also to see if any composite motifs have strong weights.

6. Ext Data Fig 7i – how can the “random Starr-seq CNN” achieve such a strong precision/recall curve? I can’t quite tell from the legend what it really is plotting (“random STARR-seq enhancer sequences”). Are these regions that have enhancer activity that were randomly selected? Are they random selections of tested enhancer sequences, regardless of Starr-seq activity? Were they from the “random DNA” library, and had enhancer activity? Are they random genomic regions? (If it is the latter, then something must be wrong with the calibration, given how strong these precision/recall curves are). Please clarify, and perhaps further emphasize what you are plotting by adding e.g. a dotted line at the random expectation level (i.e., some sort of clear baseline).

7. Fig 3b – it seems very odd that bZIP motifs have stronger activity at promoters than enhancers, given that AP-1 (a major class) is famous for being a marker of enhancers. Please clarify what type of bZIP motif this is (perhaps it is CEBP instead?)

8. How were the motifs shown in Fig 3d selected? Were these hand picked, or are you showing all motifs with some sort of positional enrichment?

9. Fig 4g – “The score indicates the fraction of predicted TSS positions falling within ± 25 bp (the area shaded with green) from the annotated TSS positions in the genome for each model separately.” How many predicted TSS positions are being made by each method? A more selective model that emphasizes specificity over sensitivity would have an advantage in this scoring system. For an extreme example, a trivial method that only makes one very strong prediction could easily achieve a perfect score of 1.

10. How certain are you regarding the conclusions of Fig 5C? I agree with the data as presented. But I am not sure that you have a large enough sequence space, even given the huge size of your library. To me, the data presented indicate that random DNA that can act as an enhancer does not generally “hit the jackpot” and activate a given promoter more than would be expected by an additive model. But this does not preclude the possibility of a small handful of enhancer DNA sequences that can. Given that evolution acts in a highly non-random manner, it still seems quite feasible that there might be many examples in the genome of enhancers with stronger than additive effects on particular promoters. (If Fig 5C is actually using genomics sequences and not random sequences, then my argument does not hold – if so, perhaps this can be clarified in the legend and text).

11. Was ‘broad peak calling’ really used for ATAC-seq, as stated in the methods? ‘Narrow’ is standard for ATAC-seq, and it looks from your browser shots that this would be more appropriate for your data

12. Very little is presented regarding the effect of DNA methylation on the Starr-seq libraries. Some results appear to be buried in Extended data Fig 7, but they are never explicitly mentioned in the main text, even at a high level. Can any conclusions be drawn from these data?

13. These cells are not being stimulated (other than the transfections themselves) – you therefore are likely missing out on a lot of stimulation/context-dependent TFs (e.g., immune system response) –

please add this to the discussion.

14. Ref 19 (de boer et al) is a very similar study to this one (although it is in yeast, not human).

Please add a comparison of the overarching conclusions of these two studies to the discussion – they represent very nice “extreme ends” of our current regulatory logic knowledge in eukaryotes.

15. Previous studies have demonstrated strong motif spacing effects, and motif dependency, on transcriptional output (e.g., early MPRA-type work from Segal lab). It is quite possible that, even with your enormous libraries, you are unable to detect these dependencies, especially if it is only true for a subset of TF pairs/sets and spacings. Please mention this as a caveat to your overall conclusions.

16. In the “Transcriptional enhancers can readily evolve de novo from random sequences” section, a convolutional neural network (CNN)-based classifier similar to DeepBind was fit to the data to “identify all sequence features”. While a CNN based classifier is a powerful tool to understand and extract useful sequence patterns, it does not necessarily extract all sequence features, which are highly dependent on the network architecture design, and its capacity. According to the CNN configurations presented in S. Table 9, the filter/kernel numbers in the first convolutional module was limited to 256, while the kernel size was limited to 5. At best, this would approximate $\sim 1/4$ of the sequence space of the same size without any probabilistic modeling. This issue needs to either be addressed in the design of the CNN, or mentioned as a caveat to any interpretations of the data.

17. In the same section, the authors discuss that analysis of the trained CNN classifier revealed that it has learned motif features similar to those identified by the logistic regression. It is not clear how the authors reached such a conclusion. The authors discussed interpretation of convolution neural network classifiers in the methods section and presented DeepLift-based visualization coupled with the mutual information (MI) analysis pipeline. But it would be more informative to directly see the aggregated sequence patterns the network has really learned by further pursuing the model interpretation analysis, e.g., by extending the work with TF-Modisco analysis following the DeepLift output.

Minor text/figure issues:

1. A more through introduction about the library design would be useful in the main text, especially for the TF motif library.

2. As written, it was unclear to me at first that the ATI experiments were performed specifically for this study (I initially thought you were comparing to the published mouse data)

3. Ext Data Fig 6 would be cleaner if it restricted to only showing TFs (looks to me like it is showing all genes)

4. The Venn Diagrams shown in Fig 2 are misleading (they do not include the entire “universe” – the versions shown in extended data fig 7g are more true to the actual data)

5. Fig 5c – (important typo) – in the figure legend, based on the text and the simple equations shown, I think you swapped the “enhancer from input” and “promoter from input” labels

6. Fig 5D – why are CREB, ETS, and NRF shown downstream of the TSS in the model? Based on Fig 4d, they should be upstream along with the TATA box

7. A public genome browser session containing your data would be very useful for the community.

8. The “Data and code availability” section currently has placeholders for code and data access – please make sure they are available upon publication

Reviewer #3:

Remarks to the Author:

This manuscript describes the results from a large Starr-seq experiment performed across multiple cell types. The experiment is designed to test combinations of TF binding sites, genomic DNA sequences,

and randomly generated sequences for regulatory activity. The primary claims of the resulting analyses are 1) that enhancers come in four distinct functional classes, 2) that only a small number of TFs are specific for each cell type, 3) that interactions between TFs do not play a large role in setting enhancer activity, and 4) that there are few if any specific interactions between enhancers and promoters.

It would be very exciting and impactful if these claims could be supported. However, as written, the manuscript does not support these claims. The text was disorganized and I could not align what was claimed in the manuscript with what was being shown in the figures. I kept thinking that there were interesting results in this paper, but I was unable to understand the rationale for the design of the experiments, what was being shown in the figures, or how the results lead to the interpretations. My only comment about the manuscript is to suggest that the authors make a much more determined effort at a clear and methodical presentation of their study.

Reviewer #4:

None

Author Rebuttal to Initial comments
--

General response to reviewers and editorial comments

In general, the referees have a positive response to our manuscript, stating that it is “*a well designed and well executed study*” (#2), as well as “*a great set of experiments and a very exciting resource*” (#1). Moreover, the referees state that our manuscript could be “*very exciting and impactful*” (#3) and “*very interesting for the community*” (#2).

The referees raise some points regarding the structure of the manuscript (#1 and #3) and interpretation of the data (#1 and #2), especially regarding the motif grammar. We have addressed all the concerns raised by the referees by re-organizing and re-writing the manuscript as well as by clarifying the text and adding new data analyses as detailed in the point-by-point response to the referees. We acknowledge that large amount of different data sets and analyses make the manuscript complex, and we have paid particular attention to making the revised version clearer for the general audience.

The main changes in the revised version include:

1. We have substantially revised the main text to improve readability and accessibility of the work to the general reader
2. We have included a new Supplementary Note with more detailed description of the rationale of the experiments and the methods used as suggested by Reviewer #1
3. We have re-structured the figure panels to make the message of the manuscript clearer
4. We have included more detailed analysis of the motif spacing and interactions to address the questions regarding motif grammar
5. We have performed new analyses to characterize in detail the motifs enriched in the STARR-seq experiments and the motifs that were learned by the convolutional neural networks
6. We have added new experimental data to address the extent of cellular alarm responses in our experimental system

We thank the reviewers for their constructive comments and feel that the revisions they suggested have significantly strengthened the manuscript. In the following pages is our point-by-point response to all specific criticisms of the reviewers. The reviewers' comments are in *italic*, and our response to them is in roman, with the changes made to the manuscript indicated in **bold**. Affected page and line(s) are also indicated.

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

In their manuscript "Sequence determinants of human gene regulatory elements" Biswajyoti Sahu et al. performed a number of very interesting reporter assays (STARR-Seq) and in vitro binding (ATI) experiments, as well as created a number of supporting RNA-seq, ATAC-seq and ChIP-seq data sets. The result is a very interesting (but also very complex) read out of regulatory elements in (mostly) two cancer cell-lines (HepG2 and GP5d). There are multiple different STARR-Seq assays in this study and the authors branch out into studying effects of methylation, the interaction of regulatory sequences ("promoter"- "enhancer" interactions) as well as the effects of a specific TF knockout (TP53). This all is supplemented by an in-depth modeling analysis using regression, gapped-kmer or CNN models.

We thank the reviewer for the interest and positive feedback to our study.

Trying to put all that into a single study comes at the cost of oversimplifying a story in the main text (without a comprehensive discussion of individual limitations) while providing a very long method description that is too complex to follow for anyone who is not acquainted with the study. My first suggestion would be to "divide and conquer" and to publish this work as a set of two to three articles in the "same issue" of a journal. I believe that we are looking at some very interesting work here, which is really not well represented the way it is currently written up. If splitting up is no option, I would highly recommend to write a structured supplemental note where the individual experiments are motivated and outlined, and most importantly their limitations discussed.

We agree that the large amount of different data sets and analyses make the manuscript complex, and we thank the reviewer for pointing out these important concerns. As suggested by the reviewer, **we have now included a Supplementary Note structured according to the main figures of the manuscript that describes the individual experiments with discussion about their limitations. In addition, the manuscript text, sub-headings, figure headings and the organization of the figure panels has been revised in order to make the manuscript clearer.**

Another high-level criticism is the availability of models and raw as well as processed data. While the authors announce that models and raw data will be released, this is something that should be done before submitting revisions of the manuscript. The reviewers need to see that all data was deposited with the appropriate meta-information.

Specifically, all data should be deposited through NCBI GEO to allow that also processed data is released. Similarly, data/methods cannot be referenced with a manuscript "in preparation" (L733). Either this manuscript is available as pre-print or the information is provided as "personal communication", in which case a sufficient description has to be provided to the reader/reviewers.

We agree that it is important to release the data, and we apologize for not having them available during the initial submission. As suggested by the reviewer, **we have now submitted the raw and processed data to GEO (GSE180158) and machine learning models to Zenodo (10.5281/zenodo.5101420). The data is currently private and will be made public upon publication of the manuscript. However, read-only access token to the GEO data will be available to the Reviewers through the Editor. Furthermore, a genome browser session with the analyzed data will be made available upon publication as suggested by Reviewer #2. The accession numbers have been updated to the revised manuscript (page 67, lines 1634-1639; page 69, lines 1682-1686).** We have also updated the

reference for TT-seq in the Methods ([page 32, line 786](#)), so the text no longer states “manuscript in preparation”.

As briefly mentioned above, the current version of the main manuscript appears to me more of a big picture story telling; while many important details are only available in the methods and a discussion of experimental limitations is missing. In this context it seems most important that even if trying to simplify for a broad readership, a correct picture is conveyed. For me issues arise for the following topics:

(1) It is essential to mention the applied experimental methods early on (i.e. in the abstract) and the authors should refrain from comparing to an "aggregated length" of tested sequences throughout the manuscript. The authors tested millions of short sequences, they never tested long sequences and a concatenated length is of no relevance here as these sequences did not act in that aggregate form.

We agree that it is important to describe the methods early in the manuscript. **To give more emphasis to the methods, we have modified Figure 1 to provide more details about different reporter libraries, experimental workflow, and data analysis types that were used in this manuscript. Moreover, more experimental details are given in the beginning of the Results section ([page 3, lines 60-64](#)) and throughout the Supplementary Note. As suggested by the Reviewer, we have modified abstract to mention the main methods ([page 1, line 23](#)), but we feel that the word limit for the abstract makes it difficult to cover the methods in more detail there.**

We thank the reviewer for pointing out the potential confusion in using the term “aggregate length” of the tested sequences. **We have revised these statements in the abstract ([page 1, line 22](#)) and the main text ([page 13, line 322](#); [page 18, line 440](#)) to better reflect the nature of the experiments** that indeed test collections of short sequences as pointed out by the reviewer but represent a sequence space that is ~ 100x larger than that of the human genome.

(2) The authors talk about "evolved" sequences, but what they mean is that they tested random sequences and that a proportion of those showed activity. This has nothing to do with molecular evolution. In some contexts, it might be sufficient to talk about motifs/sequences that are enriched/overrepresented among those activating expression.

We agree that molecular evolution typically refers to changes in sequence in response to selection pressure and apologize for using this imprecise term. **We have now changed or removed the term “evolved sequences” throughout the revised manuscript and replaced it with more accurate ones ([page 5, line 113](#); [page 6, line 132](#); [page 7, line 175](#); [page 10, lines 241, 250](#); [page 11, line 260](#); [page 13, lines 307, 315](#); [page 15, line 364](#); [page 17, line 407](#), [page 92, line 2122](#); [page 95, line 2151](#); [page 96, lines 2178, 2185](#)).**

(3a) The authors are doing an unusual normalization of STARR-Seq data, which is typically used as signal over background measure. Due to the high library complexity, sequencing the genomic background is uneconomical (L846ff) and the authors try to normalize differently (e.g. by comparing motif enrichments vs a random sample of the input). This however cannot correct for artifacts in the STARR-Seq data or for more subtle effects like GC content. The authors need to discuss these technical aspects of their data (and not just in the methods).

We thank the reviewer for the important comment and apologize if the details of the analysis of genomic STARR-seq were not clear in the original version of the manuscript. **We have now clarified on [page 8, lines 180-182](#) in the Results section of the revised manuscript that for genomic STARR-seq we**

have used standard peak caller MACS2 (as used previously for example in Peng et al., 2020, PMID: 3291229) and further discussed the analysis of genomic STARR-seq in Supplementary Note (page 6, lines 267-278). For the random STARR-seq, we analyzed enrichment of sequence features, and used *de novo* motif mining and convolutional neural network (CNN)-based methods. We agree that motif mining approach (but not CNN) could miss subtle effects like differences in mononucleotide or dinucleotide content, but we have now re-analyzed the data and confirm that these effects seem minimal since the dinucleotide frequencies follow those expected from the mononucleotide frequencies and the GC-contents of the motifs do not correlate with the motif activities. We have added these analyses to the revised manuscript (pages 44, lines 1084-1086; page 46, lines 1128-1133) and further discussion about the analysis of ultra-complex libraries to Supplementary Note (page 6, line 29–page 7, line 308). We have also clarified that we have corrected technical artifacts by removing sequences containing parts of the primer sequences or long stretches of G's that originate from the Illumina NovaSeq sequencing chemistry. **We now explain this in the revised manuscript (page 44, lines 1083-1084).**

(3b) STARR-Seq (like also MPRA/CRE-seq approaches) seem inherently biased (e.g. due to a minimal promoter) towards activating rather than repressing effects. How does this impact your study and the drawn conclusions?

We agree that MPRA approaches primarily measure transcriptional activation. We do, however, detect some repressive motifs such as OVOL1 and CUX but with weaker sensitivity, and OVOL1 was also identified as a negative feature by our logistic regression model. **These findings are now highlighted in updated Figures 2d and 5b and described in the revised manuscript (page 6, lines 137-139 and page 11, 267-269).** Most regions of the genome are not actively repressed by sequence-specific DNA binding proteins, but simply fail to activate transcription due to lack of transcriptional activators. Thus, we feel that meaningful conclusions about the active TF motifs within enhancers can be drawn from our results. However, designing experiments for measuring repressive effects would certainly be an interesting direction for future studies. **Discussion about measuring the repressive effects is also added to the Supplementary Note (page 7, lines 309-314).**

(3c) STARR-Seq assays also cause a strong cellular "immune" response and you seem to be dealing with a rather high number of plasmids per cell (L876), how does that impact your results? What about plasmid silencing?

We agree that this is a genuine concern based on the previous reports in the literature (such as Muerdter et al., 2018; PMID: 29256496) and since we also observe strong enhancer activity from the p53 and IRFs. **To address the extent of the cellular alarms in our experimental system, we performed new control experiments in which the cells were transfected with the genomic reporter plasmid library or treated with 5-fluorouracil to induce p53. Cellular responses were analyzed by RNA-seq for gene expression, ATAC-seq for chromatin accessibility, and ChIP-seq for p53 and IRF3 binding as well as for active histone mark H3K27ac.** Importantly, we found that plasmid transfection itself caused very minor changes in the cellular transcriptome or p53 and IRF binding, and gene set enrichment analysis showed that p53 target genes were strongly enriched in 5-fluorouracil-treated cells, but not in cells transfected with the STARR-seq library. These results indicate that in our system, the alarm response is very mild and the enrichment of p53 and IRF motifs in the STARR-seq experiments is not an artefact from the experimental system but a measurement of their actual enhancer activity in the cells analyzed. **These findings are shown in new Extended Data Fig. 1c-f and referred to in the revised manuscript (page 3, lines 83-85), described in the Methods (page 28, line 695–page 29 line 699;**

page 29 lines 702, 714 onwards; page 31, lines 750-752), and further discussed in the Supplementary Note (page 2, lines 53-71).

(Very incomplete list of) minor/other comments:

- L67: provide length/context of TF binding motif MPRA

The length of the sequence context has been added as suggested by the reviewer (page 3, lines 63-64 of the revised manuscript).

- L110: active TF identification (ATI) need more of an introduction

Main text has been revised to clarify the part describing ATI results (page 4, lines 92, 95-97), and more detailed introduction to the ATI method has been included in the new Supplementary Note (page 2, lines 83-97).

- L220: discuss how this relates to "primed" enhancers and whether these are active enhancers in other cell-types that only become active because they are moved into an active chromatin context.

We agree that it is an interesting question whether the enhancer classes described in this manuscript have features of the previously described primed enhancers. **We addressed this question by overlapping the peaks representing different enhancer classes with the H3K4me1 histone mark that is generally considered to mark the primed enhancers. This analysis revealed that cryptic enhancers do not show overlap with the H3K4me1 mark, but around one fourth of the closed enhancers are enriched for H3K4me1. These results are now shown in new Extended Data Fig. 9d.** However, since primed enhancers are considered to be located in open chromatin (based on e.g. Heinz et al., 2015, PMID: 25650801), closed enhancers do not fully represent the features of "primed" enhancers. **These aspects are now discussed in the Results (page 9, lines 224-227) and Supplementary Note (page 5, line 248-page 6, line 266).**

- L347: "Molecular evolution approach" -- Is there a prior definition of what this entails? I am having issues with the term evolution for this study.

We thank the reviewer for the comment. **We have now modified the statement and removed the term "molecular evolution" (page 15, line 358).**

- L401ff: The statement about TFs as basic unit makes sense and is probably not challenged. The argument whether "regulatory elements are not atomic units of gene expression" seems to be semantic one, especially as the authors argue for an additive effect of multiple TFs with some activation limit. Considering that the authors are testing these TFs in (very) close proximity (due to technical limitations), it seems an unsupported statement that there would be no length-restricted local neighborhood within the TFs act additively on expression regulation and that these should not be called regulatory elements.

We agree that the statement was too strong, and we did not intend to imply that there are no gene regulatory elements. **We have now removed this statement and re-written the sentence to clarify this (page 17, lines 419-421).**

- L522ff: Details about amount of DNA and cycles missing

Missing details have been provided in the revised manuscript (page 23, lines 546-547).

- L539ff: *Amount of material, estimate of oligo complexity*

We thank the reviewer for an important comment and apologize for the missing detail. **The amount of material and estimate of oligo complexity ($\sim 3 \times 10^{12}$ molecules) have been added to the revised manuscript (page 23, lines 567-568).**

- L558ff: *It remains unclear to me how the reporter gene is inserted in between "promoter" and "enhancer" random oligo regions, the size of 555 bp seems too short to include it. Please clarify.*

We thank the reviewer for the comment and apologize for the confusing statement. The promoter-enhancer library was cloned into a modified plasmid backbone that harbors a small *Drosophila* ORF instead of a reporter gene, as described in Methods (page 20, lines 484-487). **To indicate this also in the context of library cloning, we have clarified the statement on page 24, lines 584-585.**

- L647ff: *Template switching seems to be the required method for reading the "promoter"- "enhancer" STARR-seq library. This needs to be clearer. Also Fig4a (L1890) needs a complete description of the process. You should also bring the corresponding analysis methods closer in text (i.e. L995ff and L1010ff).*

We thank the reviewer for the comment and apologize if the usage of the template switch approach was not clear in the original version of the manuscript. **We have revised the figure legend for current Fig. 6a (page 95, lines 2149-2150) to indicate, for which figure panels the template switch data was used.** Specifically, readout from the template switch experiment was used for the analyses in which the information about the TSS positioning within the random promoter was required (i.e. in Figs 6b-d). However, template switch method is not required for other types of analyses of the promoter-enhancer STARR-seq data, such as the motif enrichment and interaction analyses shown in Figs 5 and 7. **Datasets that were used for generating different figures in the manuscript are detailed in Supplementary Table 7, and reference for that is now also provided in the legend for Fig. 6a (page 95, line 2150).** In the revised legend for Fig. 6a, we have also provided more details of the experimental workflow of the template switch method, as suggested by the reviewer (page 94, line 2144-page 95, line 2149). We have also clarified in the Methods the relationship between template switch library preparation and data analyses (page 28, lines 688-689; page 42, lines 1030-1031; page 48, lines 1183-1185).

- L729: *Description of bioinformatics analysis of data as well as of enriched motifs/known factors seems to be missing.*

We thank the reviewer for pointing out this omission. **We have now modified the Methods (page 32, lines 781-783) to describe that the data preprocessing was done as described earlier (Wei et al, 2018; 29786094) and that the analysis of enriched motifs was done as for the other data sets.** We have also mentioned in the Supplementary Note (page 2, lines 94-97) that the two most strongly enriched motifs in GP5d cells from the AT1 assay, NFI and NRF1, correspond to TFs that bind strongly in many tissues (Wei et al, 2018; 29786094).

- L830ff: *In a previous analysis you used the LFC package, why are you not using it here?*

We thank the reviewer for pointing out this inconsistency. **We have now re-analyzed this data using the LFC package and updated this information to Methods (page 36, line 882).** We have also otherwise revised and extended the activity motif position weight matrix (PWM) analysis and generated activity PWMs for other TFs as well as described in response to point 3 by Reviewer #2. The updated activity PWM for p53 is now shown in Fig. 1d and the activity PWMs for other TFs in new Extended Data Fig. 2d. The results are described in the revised manuscript on [page 4, lines 84-87](#).

- L1000: "Hamming distance of less than 3". You are probably trying to handle synthesis and sequencing errors here. Please provide more insight into why you are doing things and why you are picking these thresholds.

We thank the reviewer for the comment and admit that the rationale for choosing the threshold was not properly explained. The thresholds were chosen in such a way that it would be very unlikely to have multiple sequencing errors in one sequencing read to avoid a situation in which PCR duplicates with sequencing errors would mistakenly be considered as unique reads. **We have now described the rationale in the Methods (page 37, lines 903-905) in the context of preprocessing of random enhancer-library sequences as it is the first place where the issue comes up, and indicated this also in the section for Preprocessing of the random promoter-enhancer pairs (page 44, lines 1077-1078).**

- L1070: You are using Ensembl (L) and EPD (L1031) for TSS annotation across different analyses, you therefore need to specify the source in each case.

We apologize that we have not been clear enough when describing the TSS analysis. **We have now updated the Methods section to indicate which TSS annotation or data set was used in each analysis (page 42, line 1030; page 48, lines 1183-1185; page 49, lines 1202-1203).** Specifically, the section "Motif match positioning relative to TSS and STARR-seq vector" refers to the TSS derived from the random STARR-seq promoter library and thus the **human TSS annotation was not used there (clarified on page 48, lines 1183-1185)**, whereas in the cases where human TSS have been analyzed, the specific human TSS annotation has been used as indicated in respective sections (page 41, line 1012; page 42, line 1020; page 45, line 1114; page 65, line 1603; page 66, lines 1609, 1631).

- L1098: Why adding a pseudo count of 10?

We thank the reviewer for pointing out the missing description about adding the pseudo count. **We have now clarified this in the Methods (page 50, lines 1216-1223).**

- L1150ff: Regularization does not protect from issues with correlated features. It might slightly help the interpretation, but it does not solve the actual issue.

We thank the reviewer for the comment. **We have now clarified the text regarding the correlated features and L1-regularization in the Methods (page 52, lines 1273-1281).** In fact, LASSO regression (regression with L1-regularization) has been shown to work well with correlated features given that the regularization strength is adjusted properly (e.g. Hebiri & Lederer, 2012, arXiv:1204.1605). With a properly adjusted regularization strength, L1 norm will select only one out of a set of highly correlated features. This is in contrast to L2 norm which will favor weighting collinear features equally. Thus, we feel that L1-regularization is suitable for the analysis in this manuscript.

- L1843: *Not STARR-seq on its own, but the combination of readouts reveals the different types of regulatory elements. Especially as STARR-Seq does not show signal for some otherwise defined elements.*

We thank the reviewer for pointing out the imprecise wording. **We have now modified the figure title (page 91, line 2096) and the respective sub-heading in the Results section (page 7, line 175) to accurately reflect the analysis that was performed.**

Textual errors:

- L187: *[the] GP5d genome*
- L702: *correct sentence around "lysed"*
- L867: *which -> with*
- L1817: *delete "relatively"*

We thank the reviewer for the detailed comments regarding the manuscript text. **We have now corrected these points in the revised version of the manuscript (page 7, line 178; page 31, line 754; page 38; line 931; page 85, line 2022).**

Overall, this is a great set of experiments and a very exciting resource. I hope the authors can make all this a bit better accessible to the reader. Even as a very interested reader, I could only get through a very thin layer of this work.

We thank the reviewer for very detailed and constructive comments regarding our manuscript. We have carefully revised the manuscript to make it clearer and paid special attention to the points raised by the reviewers. We hope that the revised manuscript is more accessible to the readers.

Reviewer #2:

Remarks to the Author:

The authors present an interesting study examining the transcriptional activity of 400 billion DNA bases in three cellular contexts using STARR-seq. Collectively, their study examines ~100 times the sequence capacity of the human genome, and therefore serves as a strong platform for a large-scale unbiased view of the sequence determinants of human gene regulatory element activity.

Several key observations are presented based on the data:

- 1. There are three classes of enhancers, with different mechanisms and motif content*
- 2. Transcription factors (TFs) generally have (weak) additive effects*
- 3. Only a few TFs are strongly active in a cell type, with the strongest differences between cells representing known TFs that are important for specification and/or function of the specific lineages*
- 4. Only a subset of TFs have TSS positional dependency*
- 5. There is no evidence for 'beyond additive' interactions between promoters and enhancers*

Overall this is a well designed and well executed study. I expect it will be very interesting for the community and will spark a good amount of discussion and debate. That said, I have several comments, most of which involve data interpretation.

We thank the reviewer for the positive comments and the interest in our study.

Analysis/presentation/interpretation issues:

- 1. "The most active motifs displayed similar activities when placed in different sequence contexts, and between experiments using two different basal promoters, δ 1-crystallin and CpG-free EF1 α promoters" – not sure I totally agree with that 2nd part. For the top motifs (p53, IRF HT2 in Ext Data Fig 2B), it looks like 1000-fold vs 30-fold induction. I agree that the rankings look similar, but I would hardly call that "similar activity". I get that EF1 α is a stronger promoter, but the wording should be tweaked a bit here.*

We thank the reviewer for pointing out the imprecise wording. **We have revised the statement to indicate that the motif activities are consistent between the backbones (page 3, lines 78-79).**

- 2. How was a single TF chosen to plot the expression levels shown in Figure 1C and Figure 1D? any of those ETS motifs could correspond to several different Ets-family proteins, for example (same with NFAT/Fox/IRF/others). Something like a violin plot, which would show all possible TFs that recognize each motif, might be more informative.*

We acknowledge that assigning the enriched motif activity to a specific TF is not always possible due to the facts that many TFs bind to highly similar motifs and the binding specificities for all individual TFs are not yet known. **We have therefore updated old Fig. 1c to show expression levels of all TFs in the same structural family and moved it as new Extended Data Fig. 2a and added discussion about these limitations to the new Supplementary Note (page 1, line 33 onwards).** In addition, we have a new main figure panel (Fig. 3a), which shows the difference in mRNA levels for all TFs in two cell lines that in part can explain the observed differences in motif activity.

- 3. "As the library contained each single-base substitution to the p53 family consensus sequence, we were able to generate an activity position weight matrix (PWM) for the consensus. The activity PWM was highly similar to the SELEX derived motif for the p53 family" – can a similar exercise be performed for some other TFs? P53 has a very information-rich motif (and very high activity), so I would think it might be a bit of an outlier in terms of TF behavior in these assays.*

We agree that it is an interesting question whether PWMs for other TFs besides p53 can be derived from the measured motif activities. To address this, **we extended the analysis of motif activities with one nucleotide substitutions to other TFs as well and were able to generate activity-based PWMs for several other TFs. To highlight these results, the activity PWM for p53 is now shown in the revised main figure (Fig. 1d) and the activity PWMs for other TFs in new Extended Data Fig. 2d. The results are described in the revised manuscript on [page 4, lines 84-89](#) and further discussed in the Supplementary Note ([page 1, lines 24-32](#)).**

4. Only five de novo motifs are shown in Extended Data Fig. 5a. One of these looks like a likely 'composite element'. This is presented as evidence that "the backbone of the transcriptional system is based on individual TFs acting together without strict spacing preferences or grammar". I would need to see at least the top 40 de novo motifs in the random enhancer library, along with their p-values, predicted frequencies of occurrence, and enrichment scores to really buy this argument. This would also help bolster the results of the regression analysis.

We thank the reviewer for the important comment regarding the motif spacing. **We agree that the statement pointed out by the reviewer was too strict and thus we have removed it from the revised manuscript. To address the question regarding motif spacing and grammar, we have now performed more thorough de novo motif mining of the random enhancer STARR-seq data. The new results are shown as Extended Data Fig.6a and described on [page 5, lines 121-122](#) of the revised manuscript. We have also performed new motif matching analyses (shown in new Extended Data Figs. 5c, d, [page 9, lines 213-216](#); [page 10, lines 234-236](#)) as well as further analyses to address the motif spacing as described in the response to point 15.**

5. What happens if you use the de novo motifs in the regression analysis? I like seeing the results that use known motifs (since it is less circular), but it would be interesting to see how that compares in overall performance, and also to see if any composite motifs have strong weights.

We thank the reviewer for the comment and agree that it is an interesting question whether the *de novo* motifs can be used in the regression analysis. **To address this, we have performed logistic regression analysis of the random enhancer STARR-seq experiment using the de novo motifs. The classification performance of the de novo motif-based logistic regression classifier was similar to the HT-SELEX motif-based logistic regression (de novo logistic regression and the HT-SELEX motif-based logistic regression both obtained AUprc $\approx$ 0.55). The weights of the most important features in the de novo motif-based logistic regression are shown in new Supplementary Figure 6b and described in the Supplementary Note ([page 3, lines 131-146](#)).**

6. Ext Data Fig 7i – how can the "random Starr-seq CNN" achieve such a strong precision/recall curve? I cant quite tell from the legend what it really is plotting ("random STARR-seq enhancer sequences"). Are these regions that have enhancer activity that were randomly selected? Are they random selections of tested enhancer sequences, regardless of Starr-seq activity? Were they from the "random DNA" library, and had enhancer activity? Are they random genomic regions? (If it is the latter, then something must be wrong with the calibration, given how strong these precision/recall curves are). Please clarify, and perhaps further emphasize what you are plotting by adding e.g. a dotted line at the random expectation level (i.e., some sort of clear baseline).

We thank the reviewer for the comment and apologize for the unclear figure legend. The random STARR CNN was trained using the data from the GP5d random enhancer STARR-seq experiment. We have used balanced test sets (equal number of samples from the classes) in all binary classification tasks for

easier interpretability, meaning that area under precision-recall curve is 0.5 if class labels are assigned at random by a predictor. **We have now modified the figure legend for the current Extended Data Fig. 9e to clarify these points.**

7. Fig 3b – it seems very odd that bZIP motifs have stronger activity at promoters than enhancers, given that AP-1 (a major class) is famous for being a marker of enhancers. Please clarify what type of bZIP motif this is (perhaps it is CEBP instead?)

We thank the reviewer for pointing out the naming of the bZIP motif. **Specifically, the motif in the figure is JUN that is now indicated in the current Fig. 5b.**

8. How were the motifs shown in Fig 3d selected? Were these hand picked, or are you showing all motifs with some sort of positional enrichment?

We thank the reviewer for pointing out the missing details of the selection of the motifs shown in the figure. The motifs that are shown in the heatmap are the classical TSS-associated motifs (Initiator, TATA), the most highly enriched motifs at the promoters compared to enhancers, and generally highly enriched motifs (p53 family, YY, and CREB:MAF). **This is now clarified in the figure legend for current Fig. 6d in the revised manuscript (page 95, lines 2160-2163).** Interestingly, a very recent report has described a novel BANP motif that is enriched at gene promoters (Grand et al, 2021; PMID: 34234345). To analyze whether the BANP motif can also be detected from the *de novo* promoters enriched from the random sequences, we re-ran the analysis with BANP included. Interestingly, we observed a strong enrichment for the BANP motif at the *de novo* promoters upstream of the TSS. **These new results are shown in updated Fig. 5b, c and Fig. 6d and described on page 11, lines 264-265 and page 12, lines 301-302 of the revised manuscript.**

9. Fig 4g – “The score indicates the fraction of predicted TSS positions falling within ± 25 bp (the area shaded with green) from the annotated TSS positions in the genome for each model separately.” How many predicted TSS positions are being made by each method? A more selective model that emphasizes specificity over sensitivity would have an advantage in this scoring system. For an extreme example, a trivial method that only makes one very strong prediction could easily achieve a perfect score of 1.

We thank the reviewer for the comment and apologize for the confusing figure legend. In fact, in the analysis we made one prediction per TSS from each model, and the accuracy of these predictions was compared. **This aspect is now clarified in the figure legend (currently Fig. 6g) on page 96, lines 2180-2182.**

10. How certain are you regarding the conclusions of Fig 5C? I agree with the data as presented. But I am not sure that you have a large enough sequence space, even given the huge size of your library. To me, the data presented indicate that random DNA that can act as an enhancer does not generally “hit the jackpot” and activate a given promoter more than would be expected by an additive model. But this does not preclude the possibility of a small handful of enhancer DNA sequences that can. Given that evolution acts in a highly non-random manner, it still seems quite feasible that there might be many examples in the genome of enhancers with stronger than additive effects on particular promoters. (If Fig 5C is actually using genomics sequences and not random sequences, then my argument does not hold – if so, perhaps this can be clarified in the legend and text).

We thank the reviewer for bringing up this important aspect and we agree that genomic enhancers can be stronger than the random enhancers and have specific promoter-enhancer interactions. **To address this,**

we have now modified the Discussion on [page 18, lines 433-437](#) and added examples of such cases, such as the multi-chromosome structures that control the expression of the repertoire of olfactory receptor genes and the complex regulatory landscape of the HOX genes (refs Spitz, Nature 2019 and de Laat & Duboule, 2013, PMID: 24153303). These highly evolved genomic elements involve specificity that we did not observe in our data, suggesting that they have properties of the chromatin-dependent enhancers that STARR-seq cannot detect.

11. Was 'broad peak calling' really used for ATAC-seq, as stated in the methods? 'Narrow' is standard for ATAC-seq, and it looks from your browser shots that this would be more appropriate for your data

We thank the reviewer for the comment. Our ATAC-seq pipeline indeed uses broad peak calling, but in the genome browser snapshots, traces are shown from the BAM coverage file. **This is now clarified in the Methods ([page 31, line 762-763](#)) as well as respective figure legend ([page 91, line 2112-2113](#)) in the revised version of the manuscript.**

12. Very little is presented regarding the effect of DNA methylation on the Starr-seq libraries. Some results appear to be buried in Extended data Fig 7, but they are never explicitly mentioned in the main text, even at a high level. Can any conclusions be drawn from these data?

We agree that the results from the experiments using methylated genomic library were described in a superficial manner. **In the revised version of the manuscript, we have given more emphasis to these results by including three figure panels in the new Fig. 3 and by describing them on [page 8, lines 186-187](#) and [page 9, lines 204-207](#) and in the Supplementary Note ([page 4, line 191](#)-[page 5, line 216](#)).** Briefly, we show that cryptic enhancers are generally not silenced by DNA methylation, and that motif enrichment from the STARR-seq peaks called from the experiments using methylated and non-methylated libraries agree with the previously described methyl-plus and methyl-minus TF motifs.

13. These cells are not being stimulated (other than the transfections themselves) – you therefore are likely missing out on a lot of stimulation/context-dependent TFs (e.g., immune system response) – please add this to the discussion.

We agree that the cells in our experimental system are un-stimulated. **This is now mentioned in the new Supplementary Note ([page 2, lines 73-76](#)).** Furthermore, the plasmid transfection itself does not induce strong cellular alarm signals. We verified this by performing new control experiments where cellular responses to the plasmid transfection were analyzed using RNA-seq, ATAC-seq and ChIP-seq (see also response to Reviewer #1, point 3C). **These aspects are shown in new Extended Data Fig. 1c-f and referred to in the revised manuscript ([page 3, lines 83-85](#)), and further discussed in the Supplementary Note ([page 2, lines 53-71](#)).**

14. Ref 19 (de boer et al) is a very similar study to this one (although it is in yeast, not human). Please add a comparison of the overarching conclusions of these two studies to the discussion – they represent very nice “extreme ends” of our current regulatory logic knowledge in eukaryotes.

We thank the reviewer for this important suggestion. **We have now added a sentence in the Discussion to compare the yeast and mammalian regulatory systems ([page 15, lines 367-369](#)).**

15. Previous studies have demonstrated strong motif spacing effects, and motif dependency, on transcriptional output (e.g., early MPRA-type work from Segal lab). It is quite possible that, even with your

enormous libraries, you are unable to detect these dependencies, especially if it is only true for a subset of TF pairs/sets and spacings. Please mention this as a caveat to your overall conclusions.

We agree that the motif grammar and spacing are important questions. **Our new analysis of spacing between motif matches identified few significantly overrepresented spacing preferences for motif pairs such GRHL–ATF6. These results are now shown as new main Fig. 2c. Based on mutual information analysis, weak overall preference for motifs being relatively close (<50 bp) was also observed, as shown in new Extended Data Fig. 4e, f. We have described these findings in the Results (page 5, lines 196-202) and discussed them in the context of previous work in the Discussion (page 15, lines 363-365, 369-371) and Supplementary Note (page 3, lines 108-118).**

16. In the “Transcriptional enhancers can readily evolve de novo from random sequences” section, a convolutional neural network (CNN)-based classifier similar to DeepBind was fit to the data to “identify all sequence features”. While a CNN based classifier is a powerful tool to understand and extract useful sequence patterns, it does not necessarily extract all sequence features, which are highly dependent on the network architecture design, and its capacity. According to the CNN configurations presented in S. Table 9, the filter/kernel numbers in the first convolutional module was limited to 256, while the kernel size was limited to 5. At best, this would approximate $\sim\frac{1}{4}$ of the sequence space of the same size without any probabilistic modeling. This issue needs to either be addressed in the design of the CNN, or mentioned as a caveat to any interpretations of the data.

We thank the reviewer for pointing out this important aspect about the CNN architecture, and we agree that the statement regarding the CNN learning all the sequence features was too strong. **We have now revised the sentence and removed the claim that the CNN can learn all sequence features (page 6, line 141-142). Moreover, to acknowledge the limitations of CNN models in extracting sequence features, we have included discussion about the limit for the number of motifs/filters the CNN models can learn in the new Supplementary Note (page 3, lines 131-161).**

17. In the same section, the authors discuss that analysis of the trained CNN classifier revealed that it has learned motif features similar to those identified by the logistic regression. It is not clear how the authors reached such a conclusion. The authors discussed interpretation of convolution neural network classifiers in the methods section and presented DeepLift-based visualization coupled with the mutual information (MI) analysis pipeline. But it would be more informative to directly see the aggregated sequence patterns the network has really learned by further pursuing the model interpretation analysis, e.g., by extending the work with TF-Modisco analysis following the DeepLift output.

We thank the reviewer for pointing out that the statement regarding the CNN model learning similar motif features than the logistic regression model was not sufficiently supported by the results shown in the previous version of the manuscript. **To address this in more detail, we have now analyzed the motif patterns learned by the random enhancer CNN model in three different complementary ways, including the TF-ModISco analysis suggested by the reviewer. The discovered motif patterns are shown in new Extended Data Figs 6c, 7, and 8 and compared both against the *de novo* motifs from the random enhancer STARR-seq experiment and against the most important motif features used by the logistic regression model. This analysis shows that the CNN has learned similar motifs than the logistic regression model is using, but that there are differences especially in the lower information content regions of the motifs. These results are described in the revised manuscript (page 6, line 146-page 7, line 155) and further discussion about the new model interpretation analyses has been included to Supplementary Note (page 4, lines 162-189).**

Minor text/figure issues:

1. A more thorough introduction about the library design would be useful in the main text, especially for the TF motif library.

As suggested also by reviewer #1, **we have included more thorough introduction to the manuscript text describing the STARR-seq experiments and the used libraries (page 3, lines 60-64; Supplementary Note, page 1, lines 14-23), and, in particular, we have modified Fig. 1a to include more details about the reporter libraries and to highlight the different analyses approaches.**

2. As written, it was unclear to me at first that the ATI experiments were performed specifically for this study (I initially thought you were comparing to the published mouse data)

We apologize for not stating it clearly that the ATI experiments from GP5d cells were performed as part of this study. **The text describing the results from the ATI experiments has now been clarified (page 4, lines 92, 95-97), and more thorough introduction to the ATI method has been included in the new Supplementary Note (page 2, lines 83-97).**

3. Ext Data Fig 6 would be cleaner if it restricted to only showing TFs (looks to me like it is showing all genes)

We thank the reviewer for the comment and agree that showing only the TFs makes the message clearer. **We have now updated the figure to only show the TFs as suggested by the reviewer. The updated figure is currently shown as the main Fig. 3a and referred to on the revised manuscript on page 7, line 166.**

4. The Venn Diagrams shown in Fig 2 are misleading (they do not include the entire “universe” – the versions shown in extended data fig 7g are more true to the actual data

We agree that the Euler diagrams shown in the current Fig. 4a do not include all possible overlaps, but we feel that it is still a good way to visualize the main finding of the different regulatory element classes. **We have modified the legend for current Fig. 4a to indicate this more clearly (page 91, lines 2101-2102).** The full comparison is shown in the Extended Data Fig. 9c. and is referred to in the legend for Fig. 4a.

5. Fig 5c – (important typo) – in the figure legend, based on the text and the simple equations shown, I think you swapped the “enhancer from input” and “promoter from input” labels

We agree that the legend for the figure (current **Fig. 7c**) might have been confusing; however, the labels are correct. **To clarify the matter, we have updated the legend to state that in the “enhancer from input” model, the classification is purely based on promoter features and in the “promoter from input” purely on enhancer features (page 98, lines 2209-2210).**

6. Fig 5D – why are CREB, ETS, and NRF shown downstream of the TSS in the model? Based on Fig 4d, they should be upstream along with the TATA box

We thank the reviewer for the comment and agree that the place where the CREB, ETS, and NRF were located was confusing. **We have now modified the current Fig. 7d to indicate these factors upstream of the TSS as suggested by the reviewer.**

7. A public genome browser session containing your data would be very useful for the community.

We agree that a public genome browser session would be useful for the community, and **a genome browser session with the analyzed data will be made available upon publication (mentioned in the manuscript on [page 67, lines 1637-1639](#); [page 69, lines 1685-1686](#))**. We apologize for not having it available yet; we tried making a UCSC genome browser session to be included with this revision, but including several different data sets behind one password-protected link was not supported by the system.

8. The “Data and code availability” section currently has placeholders for code and data access – please make sure they are available upon publication

We agree that it is important to release the raw data as well as machine learning models, and we apologize for not having them available during the initial submission. As suggested by the reviewer #1, **we have now submitted the raw data and the processed files to GEO (GSE180158) and the machine learning models are available at Zenodo (10.5281/zenodo.5101420), and the Data availability statement has been updated ([page 67, lines 1634-1639](#); [page 69, lines 1682-1686](#))**. The data is currently private and will be made public upon publication of the manuscript. However, read-only access token to the GEO data will be available to the Reviewers through the Editor.

Reviewer #3:

Remarks to the Author:

This manuscript describes the results from a large Starr-seq experiment performed across multiple cell types. The experiment is designed to test combinations of TF binding sites, genomic DNA sequences, and randomly generated sequences for regulatory activity. The primary claims of the resulting analyses are 1) that enhancers come in four distinct functional classes, 2) that only a small number of TFs are specific for each cell type, 3) that interactions between TFs do not play a large role in setting enhancer activity, and 4) that there are few if any specific interactions between enhancers and promoters.

It would be very exciting and impactful if these claims could be supported. However, as written, the manuscript does not support these claims. The text was disorganized and I could not align what was claimed in the manuscript with what was being shown in the figures. I kept thinking that there were interesting results in this paper, but I was unable to understand the rationale for the design of the experiments, what was being shown in the figures, or how the results lead to the interpretations. My only comment about the manuscript is to suggest that the authors make a much more determined effort at a clear and methodical presentation of their study.

We thank the reviewer for the interest in our study. We agree that the amount of different data sets and analyses make the manuscript dense, and we apologize for providing insufficient or superficial information for following the rationale of the experiments and the conclusions drawn from the results. We have done major revisions to the manuscript with particular emphasis on making it clearer and more approachable. Main changes are the following: (1) we have included a structured Supplementary Note that describes the rationale of the experiments and the methods used in every figure as well discusses the limitations of the chosen methods; (2) we have re-organized the figure panels to have seven main figures instead of five to give more emphasis for individual analyses; (3) we have modified **Fig. 1** to have clearer presentation of the libraries as well as experimental and analysis methods used in this study; and (4) we have revised the manuscript text, including the sub-headings and figure titles to reflect the obtained results in each chapter. We hope that the revised version of the manuscript is more approachable.

Reviewer #4:

None

Decision Letter, first revision:

3rd Aug 2021

Dear Jussi and Biswa,

Thank you for submitting your revised manuscript, "Sequence determinants of human gene regulatory elements".

Due to the current lack of access to all the computer code, we are concerned that sending the manuscript back to the reviewers would lead to unnecessary delays and quite possibly an undesirable outcome of the review process.

Please see <https://www.nature.com/nature-portfolio/editorial-policies/reporting-standards> for more information on our policies regarding access to data and code.

We shall hope to receive your revised version, including separate data and code accessibility statements, as soon as possible.

Please ensure that you have completed the Reporting Summary required for review:
<https://www.nature.com/documents/nr-reporting-summary.pdf>

Please use the link below to submit a suitably revised manuscript and updated forms(s):

[REDACTED]

Best wishes,

Tiago

Tiago Faial, PhD
Senior Editor
Nature Genetics
<https://orcid.org/0000-0003-0864-1200>

Decision Letter, second revision:

24th Aug 2021

Dear Jussi,

Your Article, "Sequence determinants of human gene regulatory elements" has now been seen by the 3 original referees. You will see from their comments below that while they find your work improved, some important points are raised. We are interested in the possibility of publishing your study in Nature Genetics, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

Reviewer #1 thinks that the study has improved but still feels that it is too complex. Apart from some minor points, their main request is that you include more quantitative information throughout the text. Reviewer #2 is fully satisfied.

Reviewer #3 raises some pertinent conceptual criticisms. Like reviewer #1, they also think that the amount of data/analyses makes the paper difficult to read/interpret. Perhaps more importantly, the reviewer thinks that the broad conclusions are not supported by the data in the sense that the number of sequences analyzed is biased/limited and therefore you cannot make such strong general statements. We think that this point is well taken and warrants a careful textual revision.

We therefore invite you to revise your manuscript taking into account all reviewer comments. Please highlight all changes in the manuscript text file. At this stage we will need you to upload a copy of the manuscript in MS Word .docx or similar editable format.

When revising your manuscript:

*1) Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response may be sent back to the referees along with the revised manuscript.

*2) If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions, available http://www.nature.com/ng/authors/article_types/index.html here. Refer also to any guidelines provided in this letter.

*3) Include a revised version of any required Reporting Summary: <https://www.nature.com/documents/nr-reporting-summary.pdf>
It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review.
A revised checklist is essential for re-review of the paper.

Please be aware of our <https://www.nature.com/nature-research/editorial-policies/image-integrity> guidelines on digital image standards.

Please use the link below to submit your revised manuscript and related files:

[REDACTED]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We hope to receive your revised manuscript within eight weeks. If you cannot send it within this time, please let us know.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Genetics is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit <http://www.springernature.com/orcid>.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Tiago

Tiago Faial, PhD
Senior Editor
Nature Genetics
<https://orcid.org/0000-0003-0864-1200>

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

With the revision of their manuscript "Sequence determinants of human gene regulatory elements", Biswajyoti Sahu et al. addressed a large number of comments of three reviewers. As a result, the

main text is much more streamlined and easier to access. The methods have been extended with some missing details and the data has been prepared for public release. Further, a separate supplemental note was added to provide more motivation and a discussion of some limitations for the large number of experiments and analyses performed. This addresses my major criticism of the first round of reviews.

I still believe that the amount of material and complexity of performed analyses is too much for a single paper and that a comprehensive review of such kind of study is simply not possible. While I feel that, I have conquered a little more territory of this work while reading the updated manuscript, I will not claim that I totally got through the presented materials and analyses. As I said before, trying to put all that information into a single study comes at the cost of oversimplifying a story in the main text. It results in a very long method description that is too complex for most readers to follow and moves the discussion of individual limitations to supplementary documents that will also not be read by most readers. While I understand the pressures that underlie the publication of these massive studies, I do not think that we are doing anyone a good service with it.

I have some random notes/comments below. As indicated above, this is largely incomplete due to the amount of materials available here. There is only one point that I would like to generalize from my notes: Where possible, it would be nice to include quantitative results (number, proportions, statistical test) in the text rather than referring the reader to a figure. Examples (again an incomplete list) of that are line 189 (providing the proportion and maybe significance test), l190 (proportion of ATAC peaks predicted), l230 (number of motifs), l263-l265 (providing some numbers rather than qualitative statements like enrichment, preferential, exclusive), or l299 (enrichment values?). Here line numbers are referring to the updated manuscript and not the one with tracked changes.

Minor comments:

- l42: remove ", respectively"? it suggests that there is a clear cut difference in the presence of these marks while I believe it is more of an enrichment.
- l51: "the 1639 TFs" – approximately/at least? We still do not know all TFs and even if a reference provides a number, I would express some uncertainty.
- l61: "minimal promoter to an exon" to "minimal promoter in a reporter gene"?
- l67: "ultra-high complexity[,] reaching billions"
- l96: For me this comes not as naturally as "this suggests" would imply.
- l132: "to determine all sequence features", I would leave out the "all" as it is an absolute statement that is difficult to make.
- l426: "was found[,] is consistent"
- l1394: "external evaluation[,] we trained"
- l1633-1639: repeated later in the document

Reviewer #2:

Remarks to the Author:

The authors have done an excellent job of responding to my comments and criticisms. No further issues remain on my end.

Reviewer #3:

Remarks to the Author:

This is a revision of the manuscript from Sahu et al. describing a large set of MPRA experiments. I appreciate the authors' efforts to improve the clarity and organization of the manuscript. I still think that there is a lot of material here for one paper, and that this is making the paper difficult to understand. However, the larger problem is that the data in the manuscript do not support the unusually broad conclusions.

One claim the manuscript makes is that the study is "comprehensive", which implies that all of regulatory space has been assayed. The text makes a compelling argument that regulatory space is too big to be encompassed by sequences found in the genome, and that therefore, synthetic DNA sequences must be included. However, even though the DNA assayed here is "~100 times larger sequence space than the human genome" (line 22) when one considers "all combinations, orientations and spacings of the 1639 TFs in multiple independent sequence contexts" (lines 51-52) the sequence space assayed in this study still represents only a tiny fraction regulatory space, which undermines the claim of being comprehensive.

Another caveat related to the size of regulatory space is the claim that most TFs work independently and additively, without cooperative interactions. Since only a small fraction of regulatory space is being assayed, most combinations of TF sites, in most orientations and spacings, were not measured. Thus, the study is underpowered to detect interactions between TFs relative to the power to detect the additive contributions of TFs. No strong conclusions should be drawn from the lack of detected interactions: it is the expected result from a study with limited power to detect interactions.

Another example of a strong conclusion drawn from a negative result is on lines 95-97, where the authors invoke the sequential activity of TFs based on the lack of concordance between enhancer activities and in vitro binding data. The possibility that one or both data sets may contain significant errors is not considered.

The claim that only a small number of TFs are active in any cell type cannot be supported from assays in just two cell types, especially when both of the cell types derive from the endoderm.

The claim that there are multiple categories of enhancers is only weakly supported and comes only categorizing active sequences by their epigenetic marks. Any randomly picked group of sequences could likewise be separated by the types of marks they carry, so the mere fact that enhancers have different marks does not prove that they function in qualitatively different ways from each other.

The TFs with the largest response in the experiments are p53 and IRF, two TFs that are part of a known artifact of Starr-seq that has to do with the cellular response to foreign DNA. The authors have done experiments to rule out that the cellular alarm system is not responsible for their measured activities of p53 and IRF, but it remains worrying that this is the largest effect in the data.

Author Rebuttal, second revision:

Response to reviewers of Sahu et al.

General response to reviewers and editorial comments

In general, the referees have responded positively to the revisions we made to our manuscript. They state that *“the authors have done an excellent job of responding to my comments and criticisms”* (#2) and that the manuscript *“addresses my major criticism of the first round of reviews”* (#1). Moreover, the referees mention that they *“appreciate the authors’ efforts to improve the clarity and organization of the manuscript”* (#3) and that *“main text is much more streamlined and easier to access”* (#1).

The referees raise some further points regarding missing quantitative details (#1) and interpretation of the data (#3), especially whether the number of sequences analyzed supports the conclusions in the manuscript. We have addressed all the concerns pointed out by the referees by clarifying the text and by adding new analyses of the data, as detailed in the point-by-point response to the referees.

The main changes in the revised version are the following:

1. Addition of exact quantitative information about different analyses throughout the Results-section.
2. New analysis to estimate the power of our assay to detect true motif-motif interactions
3. New analysis to study how large effect p53 has on overall enhancer activity in the cells
4. Re-writing the text to clarify points regarding data interpretation

We thank the reviewers for their constructive comments and feel that the revisions they suggested have further strengthened the manuscript. In the following pages is our point-by-point response to all specific criticisms of the reviewers. The reviewers' comments are in *italic*, and our response to them is in roman, with the changes made to the manuscript indicated in **bold**. Affected page and line(s) are also indicated, the exact line numbers referring to the manuscript document in which the changes have been highlighted.

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

With the revision of their manuscript "Sequence determinants of human gene regulatory elements", Biswajyoti Sahu et al. addressed a large number of comments of three reviewers. As a result, the main text is much more streamlined and easier to access. The methods have been extended with some missing details and the data has been prepared for public release. Further, a separate supplemental note was added to provide more motivation and a discussion of some limitations for the large number of experiments and analyses performed. This addresses my major criticism of the first round of reviews.

We thank the reviewer for the positive feedback to our revised manuscript.

I still believe that the amount of material and complexity of performed analyses is too much for a single paper and that a comprehensive review of such kind of study is simply not possible. While I feel that, I have conquered a little more territory of this work while reading the updated manuscript, I will not claim that I totally got through the presented materials and analyses. As I said before, trying to put all that information into a single study comes at the cost of oversimplifying a story in the main text. It results in a very long method description that is too complex for most readers to follow and moves the discussion of individual limitations to supplementary documents that will also not be read by most readers. While I understand the pressures that underlie the publication of these massive studies, I do not think that we are doing anyone a good service with it.

I have some random notes/comments below. As indicated above, this is largely incomplete due to the amount of materials available here. There is only one point that I would like to generalize from my notes: Where possible, it would be nice to include quantitative results (number, proportions, statistical test) in the text rather than referring the reader to a figure. Examples (again an incomplete list) of that are line 189 (providing the proportion and maybe significance test), I190 (proportion of ATAC peaks predicted), I230 (number of motifs), I263-I265 (providing some numbers rather than qualitative statements like enrichment, preferential, exclusive), or I299 (enrichment values?). Here line numbers are referring to the updated manuscript and not the one with tracked changes.

We agree that the exact quantitative information was missing from the main text in many instances, and we thank the reviewer for pointing out this shortcoming. **We have now carefully revised the manuscript and added quantitative details throughout the revised manuscript, including the specific examples pointed out by the reviewer. All the affected page and line numbers are the following: [page 5, lines 117, 121](#); [page 6, lines 136-137, 147](#); [page 7, lines 164-165](#); [page 8, lines 198-199, 201-202](#); [page 10, lines 242-243](#); [page 11, lines 269-272, 277-280](#); [page 13, lines 314-316](#); [page 14, lines 333-335](#); [page 15, lines 365-366](#); [page 90, lines 2093-2094](#); [page 95, lines 2162-2164](#).**

Minor comments:

We thank the reviewer for detailed comments on the manuscript text. **We have modified the indicated sentences in the revised manuscript as suggested by the reviewer, and the affected page and line numbers are shown after each comment.**

- I42: remove ", respectively"? it suggests that there is a clear cut difference in the presence of these marks while I believe it is more of an enrichment.

We have modified the sentence to indicate that there is a preferential enrichment and not clear-cut distinction between the marks ([page 2, line 41](#)).

- I51: "the 1639 TFs" – approximately/at least? We still do not know all TFs and even if a reference provides a number, I would express some uncertainty.

Modified as suggested ([page 2, line 52](#)).

- I61: "minimal promoter to an exon" to "minimal promoter in a reporter gene"?

Sentence clarified (page 3, line 62).

- I67: *"ultra-high complexity[,] reaching billions"*

Modified as suggested (page 3, line 68).

- I96: *For me this comes not as naturally as "this suggests" would imply.*

We thank the reviewer for pointing out this confusing statement. **We have now clarified the text on page 4, line 95-97 and removed the confusing statement from the revised manuscript.**

- I132: *"to determine all sequence features", I would leave out the "all" as it is an absolute statement that is difficult to make.*

Modified as suggested (page 6, line 133).

- I426: *"was found[,] is consistent"*

Modified as suggested (page 19, line 453).

- I1394: *"external evaluation[,] we trained"*

Modified as suggested (page 59, line 1436).

- I1633-1639: *repeated later in the document*

Repeated text was removed from page 69.

Reviewer #2:

Remarks to the Author:

The authors have done an excellent job of responding to my comments and criticisms. No further issues remain on my end.

We thank the reviewer for the positive response to our revised manuscript.

Reviewer #3:

Remarks to the Author:

This is a revision of the manuscript from Sahu et al. describing a large set of MPRA experiments. I appreciate the authors' efforts to improve the clarity and organization of the manuscript. I still think that there is a lot of material here for one paper, and that this is making the paper difficult to understand. However, the larger problem is that the data in the manuscript do not support the unusually broad conclusions.

We thank the reviewer for the feedback on our manuscript and for appreciating the improvements that were introduced in the previous revision. We apologize if the relationship between the results and the conclusions drawn from them was not communicated well enough. We have now carefully addressed each specific concern as detailed below. We have included more quantitative information about the specific analyses throughout the manuscript as also suggested by Reviewer#1, clarified and moderated statements that we agree on re-reading the manuscript reasonably could have been perceived as overly broad and qualitative. We hope that these modifications help to clarify the relationship between the results and the conclusions.

One claim the manuscript makes is that the study is "comprehensive", which implies that all of regulatory space has been assayed. The text makes a compelling argument that regulatory space is too big to be encompassed by sequences found in the genome, and that therefore, synthetic DNA sequences must be included. However, even though the DNA assayed here is "~100 times larger sequence space than the human genome" (line 22) when one considers "all combinations, orientations and spacings of the 1639 TFs in multiple independent sequence contexts" (lines 51-52) the sequence space assayed in this study still represents only a tiny fraction regulatory space, which undermines the claim of being comprehensive.

We thank the reviewer for the comment and agree that the word "comprehensive" was too strong. **We have now toned down the statements on page 3, line 58 and page 66, line 1605 and used more appropriate words.**

Another caveat related to the size of regulatory space is the claim that most TFs work independently and additively, without cooperative interactions. Since only a small fraction of regulatory space is being assayed, most combinations of TF sites, in most orientations and spacings, were not measured. Thus, the study is underpowered to detect interactions between TFs relative to the power to detect the additive contributions of TFs. No strong conclusions should be drawn from the lack of detected interactions: it is the expected result from a study with limited power to detect interactions.

We thank the reviewer for the comment and agree that the question of the statistical power to detect specific spacings and orientations is an important one. **We have now conducted a power analysis that simulates different effect sizes and motif match thresholds with the same data size as was used in the motif spacing analysis shown in the manuscript (Fig. 2c).** The analysis shows that we do have sufficient power to detect moderate effects (fold change ≥ 3 if 1% of the pairs had a specific interaction) with high probability (> 0.77) using the same motif match threshold as in the manuscript (10^{-4}) that is consistent with the interactions shown in the figure. Moreover, even with a more stringent threshold (6×10^{-5}) that corresponds approximately to a motif with information content of 15 bits typical for TFs, we would be able to detect fold changes ≥ 5 (probability > 0.92), but only p53/p63 motif interactions were detected with this threshold. Thus, although it is difficult to rule out that some specific interactions could have been missed, the power analysis indicates that if strong specific interactions existed between individually enriched motifs in the assay, we would have detected them with very high likelihood. **These aspects are now described in the Supplementary Note (page 3, lines 111-124) of the revised manuscript.**

Another example of a strong conclusion drawn from a negative result is on lines 95-97, where the authors invoke the sequential activity of TFs based on the lack of concordance between enhancer activities and in vitro binding data. The possibility that one or both data sets may contain significant errors is not considered.

We thank the reviewer for the comment and agree that the conclusions drawn from the weak correlation between motif activity and in vitro binding data were too strong. **We have now toned down the statement in the Results section of the revised manuscript (page 4, line 95-97), and**

also added further discussion to the Supplementary Note (page 2, lines 87-89) to acknowledge the limitations in comparing the results from two different assays.

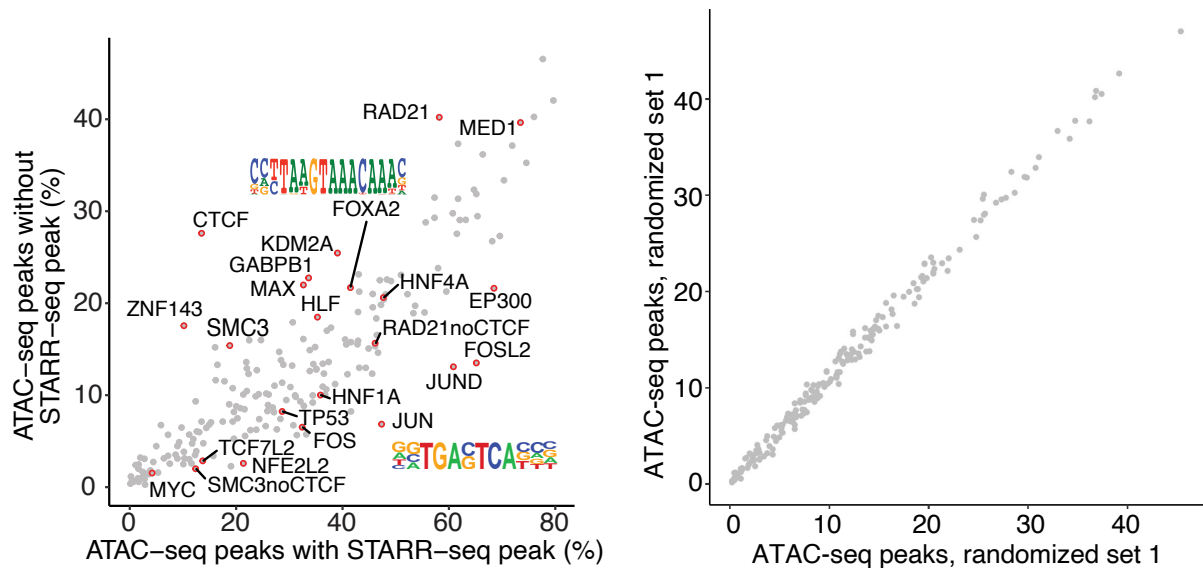
The claim that only a small number of TFs are active in any cell type cannot be supported from assays in just two cell types, especially when both of the cell types derive from the endoderm.

We thank the reviewer for the comment. In the analysis shown in Fig. 2f, we have indeed used two cell lines of endodermal origin (GP5d and HepG2). However, the binary STARR-seq assays shown in Fig. 5a were performed from three cell lines, one of which (RPE1) is of ectodermal origin. Strong correlation observed in these three cell lines is consistent with our conclusion that the active motifs enriched within the regulatory elements are largely similar in different cell types. We however agree that further studies are needed for comprehensive understanding of regulatory activity in diverse cell types. **We have now clarified this issue (page 11, lines 266-267) and state in the discussion that “further analysis using main cell types representing all three germ layers is needed to determine whether and to what extent differentiated human cell types have retained the regulatory mechanisms that existed in their common unicellular ancestor” (page 17, lines 404-406).** We also clarify in the Discussion that our result is consistent with our previous work showing that relatively few TFs show strong DNA binding activity in a cell, and that many of the strong binders are common to various cell types (Wei et al., 2018; PMID: 29786094; [page 16, line 393-page 17, line 395](#)). To further clarify the statements regarding the similarity of motif activities in different cell types, we have also now provided correlation coefficient values for Fig. 2f and Fig. 5a in the Results section of the revised manuscript ([page 7, lines 164-165; page 11, lines 271-272](#)).

The claim that there are multiple categories of enhancers is only weakly supported and comes only categorizing active sequences by their epigenetic marks. Any randomly picked group of sequences could likewise be separated by the types of marks they carry, so the mere fact that enhancers have different marks does not prove that they function in qualitatively different ways from each other.

We apologize that the results regarding different enhancer classes were not explained clearly enough. **We have now modified the statement on [page 9, lines 205-207, 211](#) to clarify that the classes were defined on the basis of the chromatin structure (ATAC-seq) and classical enhancer activity measured using a reporter assay (STARR-seq), and not only by enrichment of different epigenetic marks.** However, the enrichment for repressive histone marks was used for differentiating cryptic (silenced) enhancers from closed chromatin enhancers (both of these classes being STARR-seq+ and ATAC-seq- regions) and activating mark H3K27ac was correlated with activity of chromatin-dependent and classical enhancers. We feel that taking the epigenetic information into account is in line with how enhancer classification has been done previously (for example poised, primed, and active enhancers in Heinz et al., 2015, PMID: 25650801 were defined based on differential enrichment for H3K27me3, H3K4me1, and H3K27ac).

To further address the reviewer's concern, we also tested whether randomly picked genomic elements would show differential enrichment for regulatory features. For this, we applied a similar approach to that used for analyzing ATAC-seq peaks either overlapping or not overlapping with STARR-seq peaks (Fig. 3e in the manuscript, shown as panel on the left in **Editorial Figure 1**) for two randomized sets of ATAC-seq peaks (**Editorial Figure 1, right**). This analysis showed that there were no differentially enriched features in the two randomized sets of ATAC-seq peaks, indicating that the differential features detected when the ATAC-seq peaks were classified on the basis of STARR-seq signal arise from genuine differences between the STARR-seq+ and STARR-seq- regions.



Editorial Figure 1. Left: Fig. 3e from the manuscript that shows comparison of ChIP-seq peaks within ATAC-seq peaks with (x-axis) and without (y-axis) STARR-seq peak in HepG2 cells. For each TF or other chromatin-associated protein, the percentage of respective ATAC-seq peaks overlapping with at least one ChIP-seq peak is shown. Right: Similar analysis as in left is applied to two randomized sets of ATAC-seq peaks generated by taking the peaks having the same size as the set overlapping the STARR-seq peaks (set1) and the rest of the peaks (set 2).

The TFs with the largest response in the experiments are p53 and IRF, two TFs that are part of a known artifact of Starr-seq that has to do with the cellular response to foreign DNA. The authors have done experiments to rule out that the cellular alarm system is not responsible for their measured activities of p53 and IRF, but it remains worrying that this is the largest effect in the data.

We thank the reviewer for the important comment. We agree that from the previous version of our manuscript it may have appeared that p53 and IRF would dominate the cellular regulatory landscape. We have now performed additional analysis and clarified in the text that although p53 and IRF were the most enriched motifs, the large majority of active promoter or enhancer elements peaks do not contain these elements. As shown in Fig. 2f, p53 family motifs are the strongest activators in HepG2 and GP5d cells, having the largest fold change of motif match count over input in each cell line. To determine what fraction of all detected elements are regulated by p53, we analyzed what proportion of the genomic STARR-seq peaks overlap with TP53 ChIP-seq peaks. This analysis revealed that p53 is not responsible for most of the enhancer activity detected using STARR-seq. Specifically, 16% of the genomic STARR-seq peaks overlap with p53 ChIP-seq peak in GP5d cells, whereas only 5.1% of the STARR-seq peaks harbor a p53 motif (Extended Data Fig. 9b). In HepG2 cells, 4.9% of the genomic STARR-seq peaks overlap with p53 ChIP-seq peaks, and only 5.4% of the STARR-seq peaks harbor a p53 motif (shown in **updated Extended Data Fig. 9d**). Similarly, only 3.0% of the GP5d STARR-seq peaks harbor an IRF3 motif (shown in **updated Extended Data Fig. 9b**). **These results are now described in [page 8, lines 188-194](#) of the revised manuscript where we state that although p53 and IRF were the most enriched motifs, the large majority of active enhancers did not contain these elements. This suggests that gene regulatory system of the cell is not dominated by these cellular alarms, which is further discussed in the Supplementary Note ([page 6, line 293-page 7, line 304](#)). Certainly, the extent to which different factors contribute to the enhancer activity in a cell is an interesting question for further studies that could be addressed for example by performing the STARR-seq assay after introducing mutations throughout the genome, which is now mentioned in the Discussion of the revised manuscript on [page 17, lines 406-408](#).**

We again thank all the reviewers for their constructive comments and hope that the revised manuscript would be acceptable for publication in *Nature Genetics*.

Final Decision Letter:

Our ref: NG-A57325R2

4th Oct 2021

Dear Jussi,

Thank you for submitting your revised manuscript entitled "Sequence determinants of human gene regulatory elements" (NG-A57325R2). My colleagues and I find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Genetics, pending minor revisions to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements soon. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Genetics. Please do not hesitate to contact me if you have any questions.

Congratulations!

Sincerely,

Tiago

Tiago Faial, PhD
Senior Editor
Nature Genetics
<https://orcid.org/0000-0003-0864-1200>