

# Identifying non-coding variant effects at scale via machine learning models of cis-regulatory reporter assays

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## Abstract

The inability to interpret the functional impact of non-coding variants has been a major impediment in the promise of precision medicine. While high-throughput experimental approaches such as Massively Parallel Reporter Assays (MPRAs) have made major progress in identifying causal variants and their underlying molecular mechanisms, these tools cannot exhaustively measure variant effects genome-wide. Here we present MPAC, an ensemble of machine-learning models trained on MPRA data that provides accurate and scalable prediction of the *cis*-regulatory impact of non-coding variants. Using MPAC we predict allelic effects for 575M single nucleotide variants (SNVs) across diverse applications, including complex trait genetics, clinical and tumor sequencing, evolutionary analyses, and saturation mutagenesis. We find MPAC predictions match the performance of empirical MPRAs in identifying causal complex trait-associated alleles. We demonstrate the utility of MPAC by applying it to ClinVar, identifying non-coding pathogenic variation with higher accuracy than other sequence-to-function models. We also nominate 1,892 candidate non-coding cancer drivers by predicting the functional effects of somatic SNVs in the COSMIC database. Next, we evaluate population-level genetic variation by predicting effects for all 514M non-coding SNVs in gnomAD, quantifying the relationship between regulatory function and evolutionary constraint. Finally, we generate prospective functional maps using *in-silico* saturation mutagenesis across 18,658 human promoters, observing widespread selection against variants predicted to disrupt promoter activity. Collectively, this study establishes the value of non-coding functional predictions and provides a comprehensive, publicly available resource for variant interpretation.

## Introduction

Over 1 billion<sup>1</sup> human single nucleotide variants (SNVs) have been identified to date from rapidly growing genomic sequencing efforts<sup>2–4</sup>. As the vast majority of these variants reside in non-coding regions<sup>5–7</sup>, a major unsolved task in human genomics has been to effectively identify variants with molecular functions that ultimately impact phenotype<sup>8,9</sup>. Key examples include the over 700,000 significant associations between genomic loci and complex human diseases or traits discovered by genome-wide association studies (GWAS)<sup>10</sup>, where identifying individual causal variants remains challenging due to linkage disequilibrium (LD) with non-functional variants. Similarly, studies of the genetic underpinnings of disease driven by rare<sup>11–15</sup>, *de novo*<sup>16–19</sup>, or somatic<sup>20–23</sup> mutations have identified more variants than are tractable to experimentally study. Evolutionary constraint has been used as a proxy for function to help prioritize a subset of putatively causal variants without needing to know the underlying mechanism<sup>24–29</sup>. However, the relationship between constraint and function has not been comprehensively explored, especially in the non-coding genome.

Non-coding variants are highly enriched within *cis*-regulatory elements (CREs) such as promoters and enhancers, which precisely regulate gene expression in a spatio-temporal manner across cell types during development, homeostasis, and environmental response<sup>30,31</sup>. However, our ability to interpret single-nucleotide changes is hindered by an incomplete understanding of regulatory grammar—the complex non-linear interactions between transcription factor (TF) sequence motifs, spacing, orientation, and context that define CREs<sup>6,7,32–36</sup>. Large-scale efforts like ENCODE and others<sup>37–42</sup> have made substantial progress towards mapping the genomic locations of CREs across hundreds of cellular contexts. Association-based approaches, including molecular quantitative trait loci, have been used to map variants to function<sup>43–48</sup>, but these approaches are limited to studying naturally occurring variation. Additionally, while models trained on epigenetic state including chromatin accessibility have emerged as powerful tools for predicting CRE activity<sup>49–57</sup>, most lack training data that

includes single-nucleotide changes, limiting their applicability to predicting variant effects. Massively parallel reporter assays (MPRAs) enable high-throughput, quantitative assessment of how DNA sequences regulate transcription, with the sensitivity to detect individual variants with small effects<sup>58–60</sup>. MPRAs have been widely used to study the function of common variants from GWAS<sup>61–67</sup>, rare and somatic mutations from disease cohorts<sup>21,68</sup>, and putatively adaptive variants in human evolution<sup>69–73</sup>. However, even the largest MPRAs, which analyze hundreds of thousands of naturally occurring variants<sup>66</sup>, are outpaced by sequencing efforts that have identified over 1 billion<sup>1</sup> of the 9 billion SNVs possible in the human genome. To bridge this gap, machine learning models trained on large-scale MPRA data provide the opportunity to develop sequence-to-function models<sup>74</sup> and infer the *cis*-regulatory effects of untested variants<sup>56,75–80</sup>. We recently developed a highly accurate convolutional neural network model of CRE activity, Malinois, trained on MPRA data from 776,474 sequences, the vast majority of which represent variant alleles assayed in three cell lines<sup>78</sup>.

Here we introduce MPAC (Malinois with Parallel Aggregated Cross-validation), a scalable framework based on Malinois that is adapted for predicting changes in MPRA-measured CRE activity caused by SNVs. MPAC matches or exceeds the performance of existing variant effect prediction tools trained on larger or orthogonal datasets<sup>81–83</sup>. We demonstrate MPAC's scalability by generating hundreds of millions of predictions to identify causal and pathogenic variants, non-coding drivers of cancer, and the relationship between non-coding and coding constraint genome-wide.

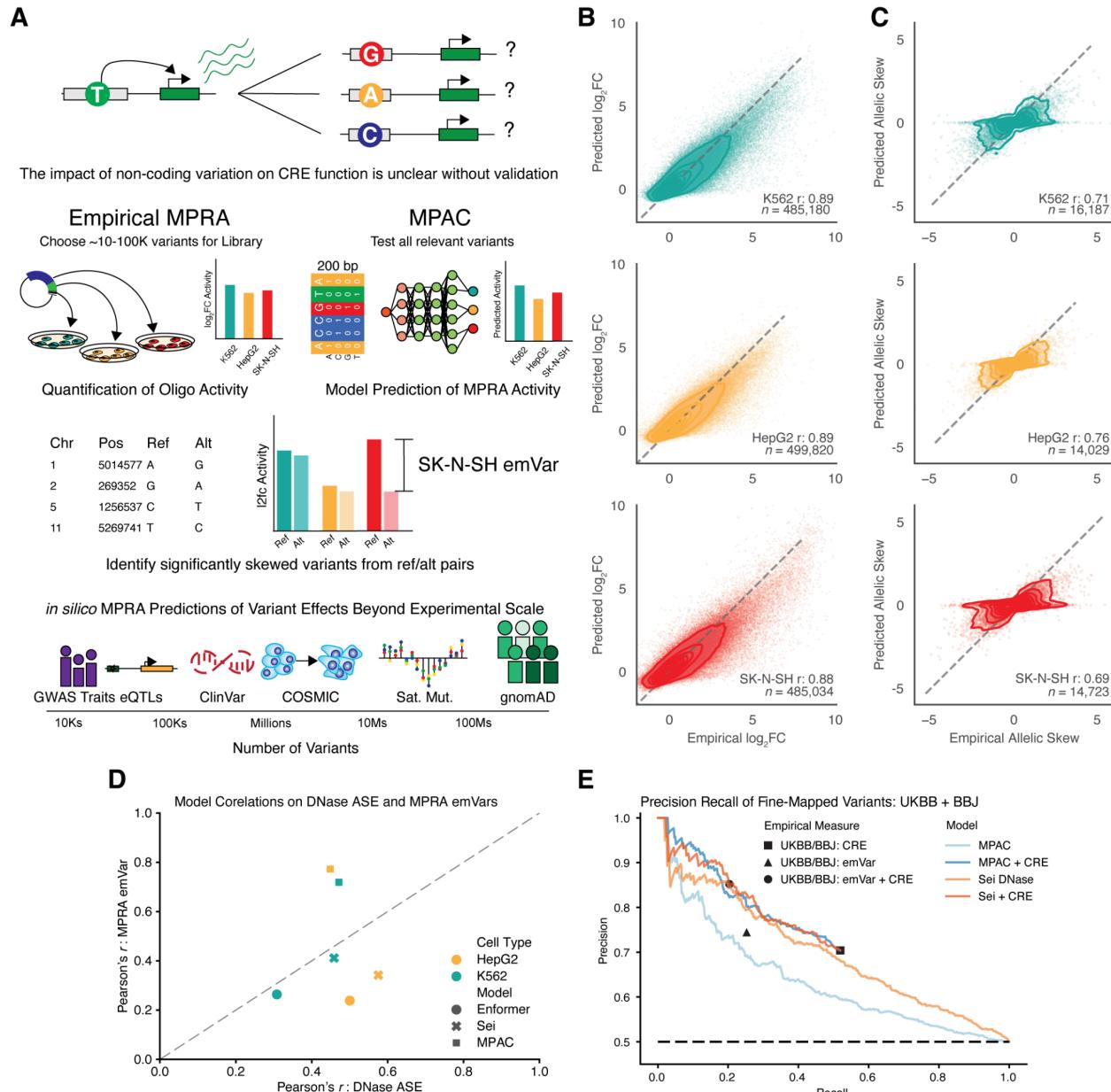
## Results

### *MPAC accurately captures variant effects of regulatory features*

To develop MPAC, we introduced several essential modifications to Malinois' CRE prediction framework, enabling genome-wide predictions of variant effects (**Figure 1A**) (**Methods**). First, we ensured unbiased CRE activity predictions for any human genomic sequence by avoiding overfitting to data seen during training. Autosomes were split into eleven pairs and each pair was used as the test set to train ten models (based on the Malinois architecture) per pair, for a total of 110 separate models (**Methods**). For any query sequence, the ten models not trained on the query's source chromosome were used for prediction and ensembled by averaging to give a final *cis*-regulatory activity ( $\log_2\text{FC}$ ) score. Across three cell types, MPAC predicts empirical MPRA activity with high accuracy (K562: Pearson's  $r = 0.89$ ,  $n = 495,180$  sequences; HepG2:  $r = 0.89$ ,  $n = 499,820$ ; and SK-N-SH:  $r = 0.88$ ,  $n = 485,034$ ) (**Fig. 1B**), and high consistency across all chromosomes: (K562:  $r = 0.88\text{--}0.91$ , HepG2:  $r = 0.88\text{--}0.91$ , and SK-N-SH:  $r = 0.87\text{--}0.90$ ) (**Supplementary Fig. 1**) (**Supplementary Dataset 1**). We further adapted MPAC for variant effect prediction by incorporating a larger sequence context, which has been shown to capture additional signals contributing to activity in reporter assays<sup>84,85</sup>. Allelic skew of a variant is calculated as the difference in predicted activity between the alternate and reference sequences, based on averaging predictions for 18 sliding 200-bp windows upstream and downstream of the variant in 10-bp increments (**Supplemental Fig. 2**) (**Methods**). MPAC allelic-skew predictions are highly correlated with empirical allelic skews for expression modulating variants (emVars) from MPRA in all three cell types (K562: Pearson's  $r = 0.71$ ,  $n = 16,187$  sequence pairs; HepG2:  $r = 0.76$ ,  $n = 14,029$ ; SK-N-SH:  $r = 0.69$ ,  $n = 14,723$ ) (**Fig. 1C**). While empirical MPRA emVars can be determined using a *p*-value threshold, we define MPAC emVars using an absolute skew threshold ( $|\text{allelic-skew } \log_2\text{FC}| > 0.5$ ), a comparable threshold to previous studies that also yields similar emVar rates<sup>66</sup>.

For a fraction (13%) of variants, MPAC-predicted and empirical allelic-skew emVar calls were discordant, defined as being an emVar in one study and non-emVar with  $|\log_2\text{FC}| < 0.05$  in the other. Discordancies were entirely due to false negative MPAC predictions. To assess if

missed predictions were due to specific sequence features or motifs, we trained a gkm-SVM model<sup>86</sup> to identify k-mers that discriminate poorly predicted emVars (**Supplementary Fig. 3**). Top scoring k-mers identified by GkmExplain<sup>87,88</sup> were enriched for AT-homopolymers and those matching known PRDM6, FOXJ3, MEF2B, LHX3, FOXQ1, and ZN770 motifs (**Supplementary Fig. 4**). However, *de novo* motifs with matches to known motifs were infrequently identified in emVars, collectively overlapping just 0.9%, 1.81%, and 4.32% of K562, HepG2, and SK-N-SH sequences respectively (**Methods**), suggesting missed MPAC predictions are largely due to sequence features infrequently seen in training.



**Fig. 1: MPAC is a highly accurate model of MPRA activity for scalable prediction of allelic skew**

**A**) Schematic illustrating the difficulty in interpreting non-coding regulatory variants and how empirical MPRAs or the predictive MPAC model can be used to identify expression modulating variants (emVars). **B)**) Scatterplot of MPAC activity predictions and empirical MPRA activity in K562 ( $n = 485,180$ ), HepG2 ( $n = 499,820$ ), and SK-N-SH ( $n = 485,034$ ). **C)**) Scatterplot of MPAC allelic skew ( $\log_2 FC$  alternate activity - reference activity) and empirical skew of MPRA emVars in K562 ( $n = 16,187$ ), HepG2 ( $n = 14,029$ ), and SK-N-SH ( $n = 14,723$ ). **D)**) Scatterplot of MPAC, Enformer, and Sei Pearson's correlation to MPRA emVars (y-axis) and DNase ASE (x-axis). **E)**) Precision-recall curves of causal complex trait-associated variation (high PIP > 0.9,  $n = 934$  vs. low PIP < 0.01,  $n = 935$ ) for CREs (black square), empirical emVars (black triangle), emVar + CRE (black circle), MPAC (light blue), MPAC + CRE (dark blue), Sei (light orange), and Sei + CRE (dark orange).

We next compared MPAC predictions to orthogonal measures of regulatory variant function (**Supplementary Dataset 2**). Although MPRA variant effects on reporter expression and DNase allele-specific effects (ASE) on chromatin accessibility capture distinct features of gene regulation, we found MPAC-predicted allelic skew and DNase ASE were significantly correlated for variants with significant DNase ASE (K562: Pearson's  $r = 0.42$ ,  $p = 3.41 \times 10^{-54}$ ; HepG2:  $r = 0.44$ ,  $p = 6.82 \times 10^{-24}$ ) (**Fig. 1D**) (**Methods**). Subsetting to variants confidently detected in both assays (MPAC emVar and DNase ASE) greatly increased correlation (K562:  $r = 0.71$ ; HepG2:  $r = 0.73$ ) (**Supplementary Fig. 5A, C**) likely by removing variants that alter chromatin accessibility independently of CRE activity<sup>89</sup>. Notably, these predicted results were comparable to correlations between empirically determined emVar allelic skew and DNase ASE (K562:  $r = 0.7$  and HepG2:  $r = 0.73$ ) (**Supplementary Fig. 5B, D**). MPAC approached or outperformed two leading sequence-to-function deep learning models on this task despite being trained on considerably less data overall and no DNaseI hypersensitivity data<sup>81,82</sup> (Sei: K562  $r = 0.46$ , HepG2  $r = 0.58$ ; Enformer: K562  $r = 0.31$ , HepG2  $r = 0.50$ ) (**Fig. 1D, Supplementary Fig. 6 and 7**) (**Methods**). Lastly, Enformer DNase ASE predictions showed low correlation to MPRA allelic skews in both K562 ( $r = 0.26$ ,  $p = 8.13 \times 10^{-190}$ ,  $n = 11,994$ ) and HepG2 ( $r = 0.24$ ,  $p = 1.55 \times 10^{-134}$ ,  $n = 10,407$ ) (**Fig. 1D, Supplementary Fig. 8**) (**Methods**) while Sei DNase ASE predictions showed modest correlation in both K562 ( $r = 0.41$ ,  $p < 10^{-300}$ ,  $n = 11,994$ ) and HepG2 ( $r = 0.34$ ,  $p = 1.99 \times 10^{-283}$ ,  $n = 10,407$ ) (**Fig. 1D, Supplementary Fig. 9**).

To assess how effectively MPAC prioritizes trait-associated causal variants, we compared its predictions against empirical MPRA measurements for 217,577 statistically fine-mapped complex trait-associated variants from UK BioBank, BioBank Japan, and eQTLs from the Genotype-Tissue Expression project (GTEx)<sup>66</sup> (**Supplementary Dataset 1**). For complex trait associations, MPAC performed comparably to empirical MPRA at identifying causal variants (posterior inclusion probability (PIP)  $> 0.9$ ) vs. non-causal variants (PIP  $< 0.1$ ) within CREs (precision = 0.82 vs. 0.85 at recall = 0.20) (**Fig. 1E**). MPAC matches Sei (AUPRCs of 0.83 for both models) (**Fig. 1E**) and outperformed the  $k$ -mer based deltaSVM model<sup>83</sup> (AUPRC 0.83 vs. 0.74) (**Supplementary Fig. 10**). For molecular traits, MPAC identified causal eQTL variants within CREs on par with empirical MPRA (precision 0.81 vs. 0.82 at empirical recall of 0.15). MPAC was slightly outperformed by Sei (AUPRC 0.78 vs. 0.81) but outperformed deltaSVM (AUPRC 0.78 vs. 0.72) (**Supplementary Fig. 11**). Overall, MPAC is able to prioritize candidate causal variants with precision comparable to empirical MPRA, at vastly reduced cost, time, and labor.

### *MPAC discerns clinically relevant germline and somatic variants*

The inability to quickly categorize non-coding variants as pathogenic or benign limits our understanding of their contribution to rare or Mendelian diseases<sup>90,91</sup>. Since CREs, as determined by DNase I Hypersensitivity Sites (DHS), show weak enrichment for pathogenic variants compared to non-CRE regions ( $OR = 1.22$ ,  $p = 0.02$ ), we sought to determine whether MPAC predictions provide better discrimination. We applied MPAC to all 180,032 non-coding variants in the ClinVar database (**Figure 2A**) (**Methods**) (**Supplementary Dataset 3**) and found that MPAC emVars exhibited greater enrichment for pathogenic variants when considering all non-coding ClinVar variants ( $OR = 3.48$ ,  $p = 2.25 \times 10^{-11}$ ) (**Supplementary Fig. 12A**), increasing further when restricting to CREs ( $OR = 12.09$ ,  $p = 4.77 \times 10^{-15}$ , Fisher's exact test) (**Supplementary Fig. 12A**). We next compared the precision and recall performance of MPAC with Sei's DHS allelic-effect predictions using matched sets of pathogenic and benign variants (**Methods**). Across all non-coding ClinVar variants, both MPAC and Sei performed well, with MPAC achieving a mean AUPRC of 0.65 and Sei 0.61. Performance improved to 0.68 (MPAC) and 0.64 (Sei) when restricting variants to DHS regions, and further increased within promoters (MPAC: 0.82; Sei 0.78) (**Fig. 2B, Supplementary Fig. 12B**).

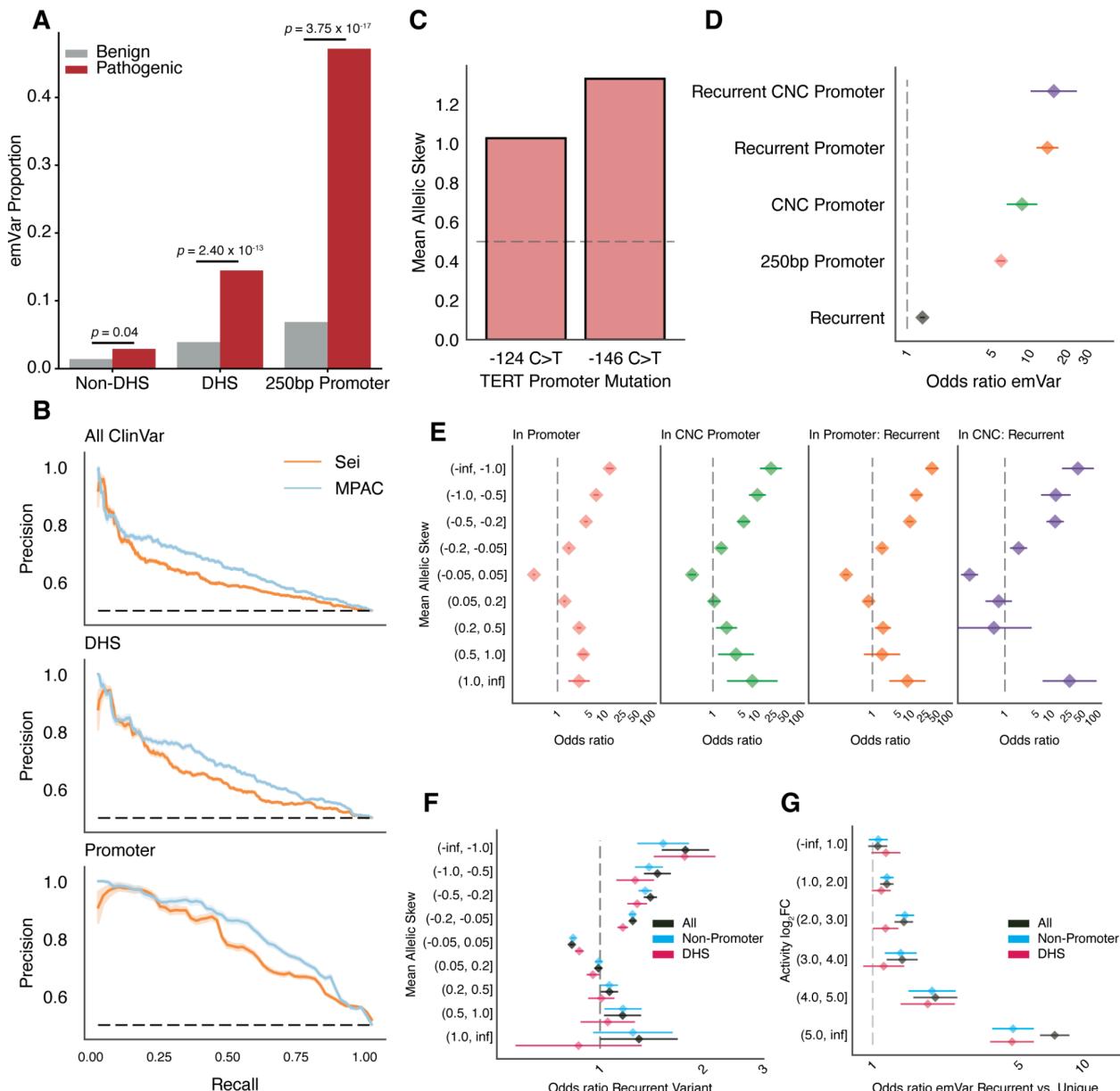
Individual tumors can harbor tens to hundreds of thousands of somatic mutations<sup>92,93</sup>, the majority of which are likely non-functional passenger mutations, making the identification of driver mutations experimentally intractable. Large sequencing cohorts can infer variant function by identifying mutations that recur across patients, as such recurrence is unlikely to occur by chance. However, non-coding mutations have proven difficult to identify<sup>94</sup> with only a few identified as important for tumorigenesis<sup>22,93,95,96</sup>. A well-characterized example of recurrent, non-coding driver mutations are those reactivating TERT by creating ETS-family TF binding sites in its promoter<sup>95-97</sup>. These two known mutations, -124 (C>T) and -146 (C>T) were predicted by MPAC to be emVars, increasing expression (-124 C>T: mean allelic skew = 1.03 and -146 C>T: mean allelic skew = 1.33) (**Fig. 2C**). Globally, ETS-family binding sites have been shown to be enriched for somatic mutations at promoters using a TF-aware burden test and functional validation<sup>98</sup>. Using MPAC, we detected 61% of the ETS-family binding putative non-coding driver mutations as emVars, supporting our *in silico* approach to nominate variants associated with tumorigenesis (**Methods**).

Encouraged by our validation of known TF binding sites, we assessed emVar enrichments for somatic mutations more broadly in the Catalog of Somatic Mutations in Cancer (COSMIC v98) (**Supplementary Dataset 4**). We observed that across all promoters, variants within 250 bp of a transcription start site (TSS) were more likely to be emVars than those in distal regions ( $OR = 5.95, p = 2.34 \times 10^{-288}$ ) (**Fig. 2D**). When subsetting to the promoters of genes previously associated with cancers from the Cornell Non-Coding Cancer Driver Database (CNC) (**Methods**)<sup>99</sup> this enrichment increased ( $OR = 8.86, p = 4.13 \times 10^{-31}$ ). Notably, regardless of inclusion in CNC, variants in promoters showed the greatest enrichment for strong effect mutations (allelic skew  $\leq -1$  or  $\geq 1$ ) (**Fig. 2E**), with a bias towards loss-of-function (LoF) mutations when considering all promoters (**Fig. 2E**). Finally, when comparing mutations that occur across multiple individuals to those appearing once, we observed that recurrent variants in promoters were significantly more likely to be emVars ( $OR = 14.35, p = 6.81 \times 10^{-84}$ ). Recurrent promoter variants for CNC genes displayed the highest emVar enrichment ( $OR = 16.18, p = 9.18 \times 10^{-22}$ ) (**Fig. 2D**). We next considered all 2,432,725 variants in COSMIC to determine whether MPAC identified functional enrichments beyond promoters. Across the entire dataset, recurrent variants were enriched for MPAC-emVars compared to variants observed in only one individual ( $OR = 1.34, p = 9.76 \times 10^{-32}$ ) (**Fig. 2D**). When recurrent and unique variants were partitioned by mean allelic skew, we identified enrichments for large negative skew variants for all recurrent variants, variants outside of promoters, and those within DHS elements (allelic skew  $\leq -1$ ) (**Fig. 2F**). Furthermore, we expect recurrent driver mutations to be enriched in sequences with high predicted CRE activity. Activity predictions from MPAC correlated with DHS element likelihood; notably, 98% of emVars with a mean reference  $\log_2FC > 5$  overlapped DHS elements. We observed enrichment between active sequences (all bins  $\log_2FC > 1$ , All COSMIC variants) and recurrence ( $OR = 1.17, p = 4.41 \times 10^{-5}$ ), which is greatest at highly active sequences ( $\log_2FC > 5, OR = 7.39, p = 8.62 \times 10^{-85}$ ) (**Fig. 2G**). This enrichment persisted after excluding promoters both genome-wide and within DHS peaks (**Fig. 2G**). In total, we nominated 117 promoter and 1,775 non-promoter recurrent emVars as candidate non-coding drivers across COSMIC.

### *Scaling MPAC predictions to common and rare human genetic variation reveals patterns of functional constraint*

To systematically evaluate the functional consequences of rare and common variation from globally diverse human populations, we first used MPAC to assess the regulatory activity of 514 million non-coding SNVs from the 76,156 genomes in The Genome Aggregation Database (gnomAD v3.1.2) (**Supplementary Fig. 13**) (**Methods**) (**Supplementary Dataset 5**). We observed that 10.0% (51.5M) of all variants were predicted to be in active sequences ( $\log_2FC >$

1). We intersected all gnomAD variants with ENCODE candidate CREs (cCREs) which cover 7.9% of the genome<sup>37</sup>, identifying 0.4% (1.9M) in promoter-like signatures (PLS), 2.1% (10.9M) in proximal enhancer-like signatures (pELS), 14.3% (73.6M) in distal enhancer-like signatures (dELS), 5.7% (29.4M) in additional cCREs such as TF binding sites (other cCREs), and 77.5% (398M) in regions outside of cCREs (non-cCRE) (**Fig. 3A**). As expected, variants overlapping PLS showed the highest average predicted activity ( $\log_2 FC = 2.26$ ), followed by pELS ( $\log_2 FC = 0.65$ ) and dELS ( $\log_2 FC = 0.48$ ), while variants outside of cCREs showed minimal activity ( $\log_2 FC = 0.25$ ), suggesting that MPAC precisely captures the activity difference of cCRE annotations (**Fig. 3B**).

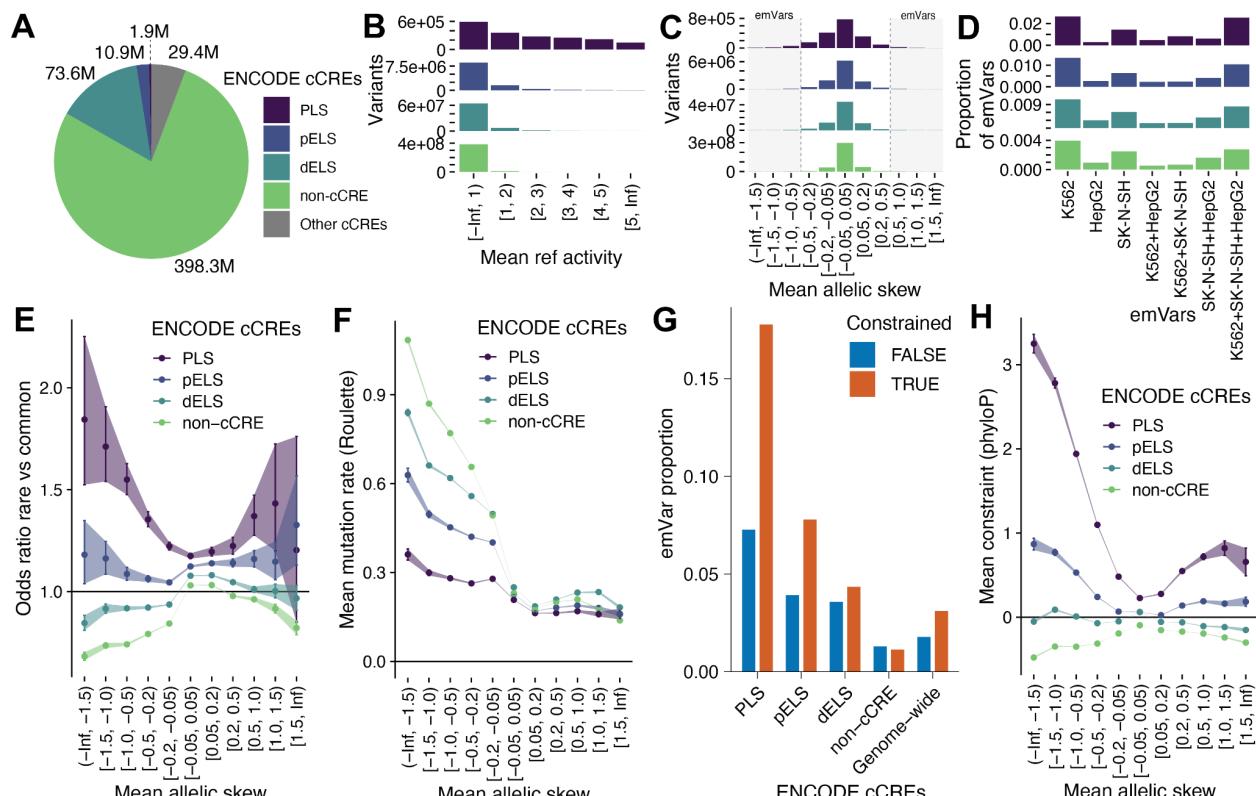


**Fig. 2: MPAC identifies non-coding pathogenic variants and recurrent cancer drivers**

**A)** Bar plot showing the proportion of ClinVar pathogenic and benign emVars in different genomic contexts. DHS: DNase I hypersensitive site.  $p$ -values from Chi-Square Test. **B)** Precision-recall curves of ClinVar pathogenic vs. matched downsampled benign non-coding variants (All ClinVar:  $n = 573$ ; DHS:  $n = 228$  pathogenic, 228 benign; Promoter:  $n = 53$  pathogenic). **C)** Mean allelic skew of TERT promoter mutations. **D)** Odds ratio (OR) that a variant is an emVar in COSMIC subsets. **E)** OR that a variant is in promoter annotations by mean skew bin. **F)** OR that a variant is recurrent compared to unique binned by mean skew. **G)** OR that a recurrent variant is an emVar compared to unique variants binned by sequence activity. Error bars in panels D-G represent 95% CIs.

Next, we used MPAC to predict allelic skew for all gnomAD variants<sup>65,66,80,100</sup>. MPAC predicted 9.4M (1.8%) of all variants to be emVars, consistent with rates in empirical MPRA studies<sup>61–63</sup>. As expected due to their overall higher constraint, PLS contained the highest proportion of emVars (8.83%) (**Fig. 3C**) and displayed the largest absolute allelic skews (PLS vs. genome-wide mean  $|\log_2\text{FC}| = 0.139$  vs. 0.057) (**Fig. 3C**). Consistent with promoters' more ubiquitous function, cell type specificity of emVars was lowest in PLS (49.5%), but increased in contexts farther from the TSS (pELS: 53.7%, dELS: 55.5%) (**Fig. 3D**), consistent with the established role of distal CREs in driving cell type-specific gene expression (**Supplementary Fig. 14**)<sup>101,102</sup>. As a resource, we also provide allelic-skew predictions for lead SNVs and variants in high LD ( $r^2 \geq 0.7$ ) for SNVs found in the 5,781 studies in the GWAS Catalog<sup>10</sup> (**Supplementary Dataset 6**) (**Methods**). Across all trait-associated SNVs assessed, 1.4% (33,808 out of 2,415,078) are predicted to be emVars and 24.6% (184,663 out of 750,574) of trait-associations have at least one predicted emVar. Lead SNVs were more likely to be emVars than SNVs in LD ( $OR = 1.31\text{--}1.43$  across populations,  $p < 10^{-31}$ ).

As CRE activity is largely defined by the combinatorial binding of transcription factors (TF) to accessible chromatin<sup>35,103</sup>, we hypothesized emVars should be enriched at accessible and TF-bound regions. gnomAD variants with large predicted effect sizes, defined as having an allelic skew  $|\log_2\text{FC}| > 1.5$ , were highly enriched in DNase I TF footprints<sup>104</sup> (negative:  $OR = 8.70$ , positive:  $OR = 3.11$ ) and TF ChIP-seq peaks<sup>105,106</sup> (negative:  $OR = 2.31$ , positive:  $OR = 2.12$ ) (**Supplementary Fig. 15A,B**). Conversely, variants predicted to have little to no impact (allelic skew  $|\log_2\text{FC}| < 0.05$ ) were depleted for TF binding (TF footprints:  $OR = 0.37$ , TF ChIP-seq:  $OR = 0.62$ ). However, the vast majority of emVars were located outside of annotated



**Fig. 3: MPAC-predicted emVars in gnomAD are under strong purifying selection**

A) Overlap of non-coding gnomAD SNVs with ENCODE cCRE classes including PLS, pELS, dELS, other cCREs, and non-cCRE regions. Distribution of gnomAD SNVs across cCRE classes based on **B**) mean MPAC activity ( $\log_2\text{FC}$ ) predictions and **C**) mean MPAC allelic-skew ( $\log_2\text{FC}$ ) predictions. **D**) Proportion of expression-modulating variants (emVars) in one or more cell lines by cCRE classes. **E**) Recent purifying selection, quantified by the OR of rare (MAF < 0.1%) vs. common (MAF > 0.1%) SNVs relative to genome-wide baseline, across cCRE classes with different levels of mean MPAC allelic-skew predictions. **F**) Mean mutation rates, as predicted by Roulette. **G**) Proportion of variants called emVars at constrained and unconstrained bases in different cCRE classes ( $\text{phyloP} > 2.27$ ,  $\text{FDR} < 0.05$ ). **H**) Evolutionary constraint of gnomAD SNVs, measured by mean Zoonomia phyloP score. Error bars in panels E-G represent 95% CIs.

TF footprints (85.9%, 3.77M vs. 617k) or TF ChIP-seq peaks (92.1%, 4.04M vs. 346k) (**Supplementary Fig. 15C,D**), highlighting the limitations of current TF binding annotations. Non-coding functional variants are likely to be deleterious and thus under constraint within-species<sup>107–109</sup>. However, this relationship has only been explored with MPRA for a small subset of CREs<sup>110</sup>, as most past studies ascertained variants biased by their trait associations. We stratified all observed gnomAD variants by MPAC allelic-skew predictions and used the odds ratio (OR) of rare SNVs (allele frequency (AF) ≤ 0.1%) vs. common SNVs (AF > 0.1%) as a proxy for the overall strength of recent purifying selection. Starting with variants in PLS, we observed higher constraint at extremes of allelic skew compared to variants with less or no allelic skew (**Fig. 3E, Supplementary Fig. 16A-C**). This U-shaped relationship was asymmetric, with large negative allelic-skew variants displaying the strongest purifying selection (OR = 1.84) compared to those with minimal allelic skew ( $|\log_2\text{FC}| < 0.05$ , OR = 1.17). While this pattern is consistent with purifying selection against deleterious promoter loss-of-function mutations, mutations with large positive allelic skews also displayed purifying selection (OR = 1.20). We see a similar pattern for variants overlapping pELS, but not dELS or non-cCRE contexts (**Fig. 3E**). Lastly, we found that the predicted *de novo* mutation rate increases with the magnitude of negative allelic skews (**Fig. 3F, Supplementary Fig. 16D-F**). This trend was strongest for variants outside cCREs, and within cCREs was strongest for more promoter-distal cCREs.

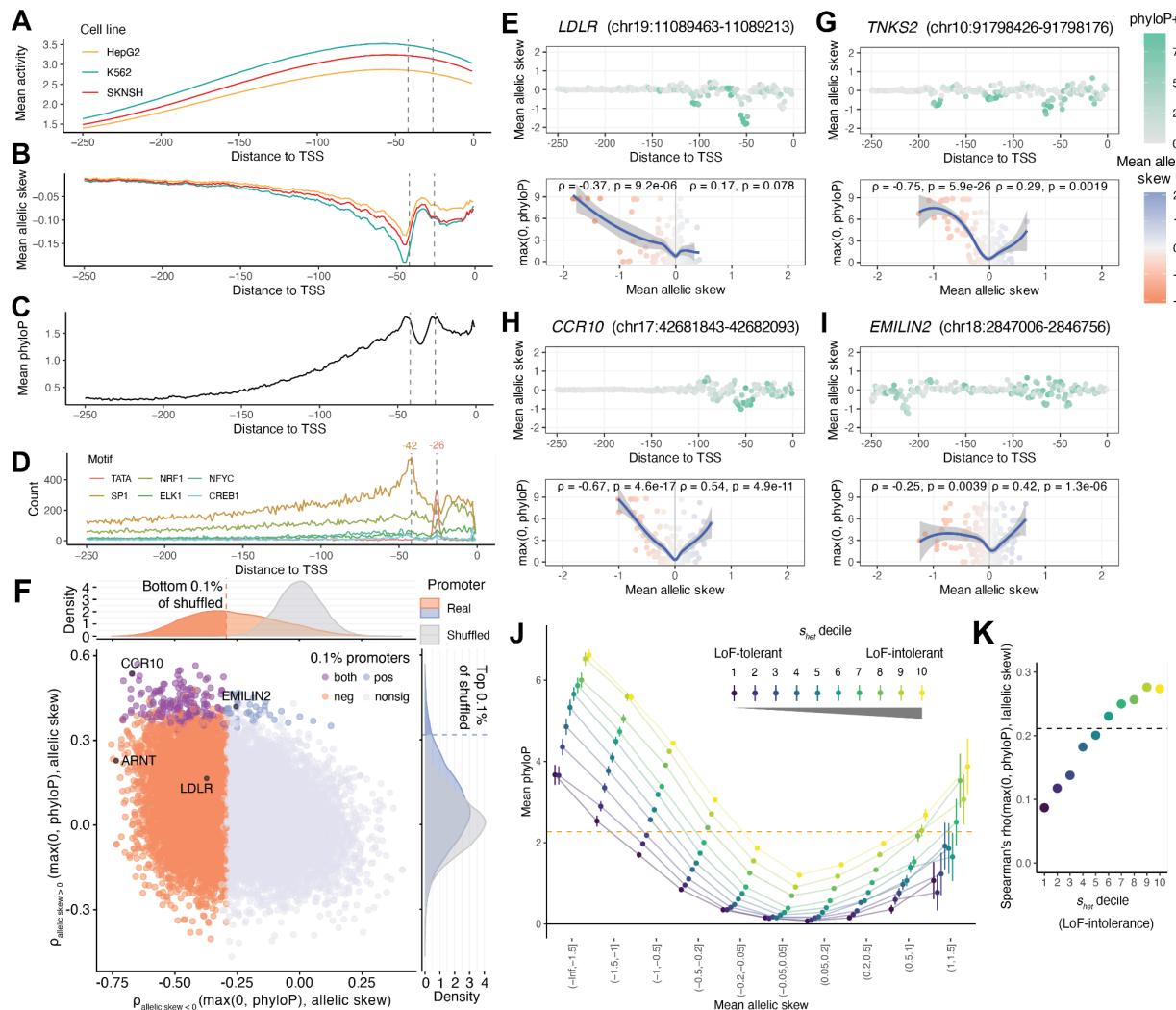
#### *MPAC enables a comprehensive exploration of the evolutionary constraint-function relationship*

Variant effect predictors have used evolutionary constraint across species as an indirect measure to assess potential pathogenicity, but this relationship has not been comprehensively evaluated for non-coding regions<sup>111,112</sup>. To evaluate if MPAC could effectively identify constrained, functional regulatory variants, we compared predicted emVars to evolutionary constraint maps at base-level resolution from the Zoonomia 241-way mammalian alignment which defines constrained bases as having phyloP > 2.27 (FDR < 0.05)<sup>26</sup>. As expected, within known cCREs, gnomAD variants overlapping constrained bases were significantly more likely to be emVars than those overlapping unconstrained bases (OR = 1.77,  $p < 2.2 \times 10^{-16}$ , Fisher's exact test) (**Fig. 3G**). Across cCRE classes, the highest enrichment was found in promoters (PLS: OR = 2.76, pELS: OR = 2.07, dELS: OR = 1.22, and non-CRE: OR = 0.87, all  $p < 2.2 \times 10^{-16}$ ) (**Fig. 3G**). However, among the 322,644 emVars with evidence of constraint, the majority were found in pELS and dELS (65.2%) compared to PLS (15.1%), reflecting their larger portion of the genome (**Supplementary Fig. 17**).

MPAC's quantitative predictions further allowed us to examine how the magnitude and directionality of variant function relates to evolutionary constraint. We compared phyloP scores to MPAC allelic-skew predictions across cCRE classes. Similar to our findings using within-species constraint, variants in PLS exhibited a U-shaped relationship between cross-species constraint and allelic skew (**Fig. 3H, Supplementary Fig. 18A-C**). Variants with large negative allelic skew displayed very strong levels of cross-species constraint (mean phyloP = 3.25), much higher than those with no allelic skew (mean phyloP = 0.23). Comparatively, variants with large positive allelic skew also showed increased constraint, albeit more modest (mean phyloP = 0.66). The constraint of the most negatively impactful variants was slightly higher than that of start loss variants, splice donor, and splice acceptor variants (mean phyloP = 3.14, 2.87, 2.85, respectively) and almost as high as that of missense and stop gained variants (mean phyloP = 3.61, 3.45, respectively) (**Supplementary Fig. 18D**). Thus, MPAC can prioritize non-coding variants with functional impacts as deleterious as coding mutations.

We next looked at non-promoter regions, which prior studies have shown display a weaker relationship between function and constraint<sup>113–115</sup>. Consistent with these findings, we

detected a similar but weaker U-shaped relationship between constraint and function in pELS regions, with large negative and positive allelic skews under more constraint ( $\text{phyloP} = 0.87, 0.18$ ) than those with minimal effects ( $\text{phyloP} = 0.07$ ). Comparatively, we did not observe this pattern for variants in dELS (Fig. 3H). Similarly, we observed that pleiotropy, as measured by sharing of emVars across cell types, increased with constraint in PLS and pELS, but not dELS (Supplementary Fig. 19A). Notably, MPAC activity of the element harboring the variant did not correlate strongly with constraint (Supplementary Fig. 19B), underscoring the importance of variant-level functional predictions. The overall weaker relationship between function and constraint at non-promoter CREs is consistent with their greater evolutionary turnover<sup>116</sup> and functional redundancy resulting in phenotypic robustness<sup>117</sup>.



**Fig. 4: Saturation mutagenesis of 18,658 promoters reveals interplay between variant function, LoF tolerance, and constraint**

Meta-promoter plots of mean MPAC-predicted **A**) activity ( $\log_2\text{FC}$ ) and **B**) allelic skew ( $\log_2\text{FC}$ ) from in silico saturation mutagenesis of the 250-bp promoter region upstream of the TSS of 18,658 canonical protein-coding genes. **C**) Meta-promoter plots of evolutionary constraint (mean phyloP). **D**) Motif counts by distance to TSS of representative upstream promoter-binding transcription factors TATA-binding protein, NRF1, NFYC, SP1, ELK1, and CREB1. Vertical lines correspond to modes of TATA and SP1 motif counts. **E**) Promoter-level plots of mean allelic skew by distance to the TSS colored by  $\max(0, \text{phyloP})$  (top) and the correlation ( $\rho$ ) and p-values of  $\max(0, \text{phyloP})$  and allelic skew for mutations with negative (bottom left) or positive (bottom right) allelic skew for *LDLR*. **F**) Promoter-level correlations ( $\rho$ ) between constraint ( $\max(0, \text{phyloP})$ ) and MPAC allelic-skew predictions for the subset of SNVs with negative (x-axis) or positive (y-axis) allelic skew. Colors highlight promoters with  $\rho$  in bottom 0.1% for negative allelic-skew mutations (red) or top 0.1% for positive allelic-skew mutations (blue) relative to permutation-based nulls. Plotting the subset of 16,995 promoters for which both correlations are well-defined (see Methods). Example promoters with strong correlations between phyloP and allelic skew for **G**) negative allelic-skew mutations only, *TNKS2*; **H**) both negative and positive allelic-skew mutations, *CCR10*; and **I**) positive allelic-skew mutations only, *EMILIN2*. **J**) Relationship between promoter variant function (mean allelic skew) and constraint (mean phyloP) stratified by gene-level loss-of-function (LoF) intolerance ( $s_{\text{het}}$  decile). Error bars represent 95% CIs. Constraint cutoff ( $\text{phyloP} = 2.27$ ,  $\text{FDR} < 0.05$ ) shown as orange line. **K**) Spearman's  $\rho$  between variant function (allelic skew) and constraint ( $\max(0, \text{phyloP})$ ) by  $s_{\text{het}}$  decile and genome-wide (dashed line).

While purifying selection prevents deleterious variants from reaching high allele frequencies, most variants are expected to be non-functional and the majority of variants in gnomAD are rare ( $AF < 0.1\%$ ). Evolutionary constraint has a moderate ability to distinguish rare from common variation ( $\text{phyloP} = -0.30$  vs  $-0.08$ ) (**Supplementary Fig. 20A**), but the constraint threshold distinguishing functional from non-functional regulatory variants is unknown. We hypothesized that stratifying variants by their regulatory impacts could decompose this relationship. As expected, across all allelic-skew bins, constraint increased with allele rarity; however this relationship varied substantially across different regulatory contexts (**Supplementary Fig. 20B**). Variants in PLS showed the strongest signals of purifying selection, with the highest constraint observed for singleton variants with large negative allelic skew ( $\text{phyloP} = 3.59$ ) compared to either singletons with minimal predicted skew ( $\text{phyloP} = 0.31$ ) or all singletons without functional stratification ( $\text{phyloP} = 0.56$ ). For ultra-rare PLS variants, constraint was similarly much higher at functional positions ( $\text{phyloP} = 3.09$ ) compared to non-functional ( $\text{phyloP} = 0.23$ ). Variants in pELS displayed a similar but more modest pattern, whereas variants in dELS and those outside of cCREs consistently showed the lowest constraint and weakest relationship with allele frequency.

#### *Prospective in-silico saturation mutagenesis of all promoters*

We deployed MPAC to prospectively and comprehensively map the regulatory impact of all possible SNVs in human promoters genome-wide (**Supplementary Dataset 7**). We generated MPAC predictions for all three non-reference single nucleotide mutations within the promoter of 18,658 protein-coding genes, defined as positions -1000 bp to -1 bp upstream of the canonical TSS (**Methods**). Most (53.4%) emVars are clustered within the proximal 250 bp upstream of the TSS, where they displayed both higher mean activity and greater mean allelic skew than emVars found in more distal regions (**Supplementary Fig. 21A,B**). Interestingly, two-thirds (67.2%) of emVars are predicted to reduce promoter activity (mean allelic skew = -0.64) instead of increasing activity (mean allelic skew = 0.34), suggesting functional promoter mutations are more likely to be disruptive. Activity and allelic skew were most correlated across cell types in the 250 bps proximal to the TSS (activity: Spearman's  $\rho = 0.93$ - $0.97$ ; allelic skew:  $\rho = 0.74$ - $0.83$ ) (**Supplementary Fig. 22A-D**), and decreased with distance. Moreover, constraint ( $\text{phyloP}$  score) (**Supplementary Fig. 21C**) and correlation between constraint and allelic skew (**Supplementary Fig. 22E-H**) were highest proximal to the TSS, consistent with the central role of the core initiation site. Focusing on the core promoter (proximal 250 bp to TSS), we next sought to understand if MPAC predictions captured known features of promoter initiation. Across all three cell types, we observed high predicted regulatory activity broadly from -150 to 0 bp from the TSS (average  $\log_2\text{FC}$  across all promoters and cell lines) (**Fig. 4A**), suggesting sequences in this region may be particularly sensitive to mutations. Within this range, we found two regions particularly concentrated in positions of large allelic skew (average  $\log_2\text{FC}$  of three possible mutations across all promoters and cell lines) (**Fig. 4B**) and coinciding with elevated evolutionary constraint, suggesting underlying sequence features drive these patterns (**Fig. 4C**). We next asked if known promoter motifs<sup>118</sup> might be disrupted at these regions, identifying broadly negative allelic skew extending from the TSS to -26. As expected, this region harbors core promoter elements for the TATA box, NRF1, and ELK1 (**Fig. 4D, Supplementary Fig. 23**). Further upstream, we observed a strong skew peak centered at -45 that co-localised with the promoter-associated TF SP1 motif (**Fig. 4D, Supplementary Fig. 23**). We also found that predicted activity of promoters was significantly correlated with gene expression levels in all three cell types (Pearson's  $r = 0.59$ - $0.53$ , all  $p < 2.2 \times 10^{-16}$ ) (**Supplementary Fig. 24**). Together, these findings show the ability of MPAC saturation mutagenesis to recapitulate known aspects of promoter regulatory grammar.

Saturation mutagenesis MPRAs have previously observed variable correlations between constraint ( $\text{phyloP}$ ) and variant function (empirical allelic skew) from studies of a limited number

of promoters<sup>59,66,119,120 27,119</sup>, so we explored the strength and generality of this relationship across all promoters. At the previously examined *LDLR* (low-density lipoprotein receptor) promoter<sup>27,119</sup>, we separately compared negative LoF and positive gain of function (GoF) allelic-skew mutations to constraint and confirmed the previously observed correlation. Notably, the correlation was driven by negative allelic skew (Spearman's  $\rho = -0.37$ ,  $p = 9.2 \times 10^{-6}$ ) (**Fig. 4E**) with no significant correlation between phyloP and positive allelic-skew mutations ( $\rho = 0.17$ ,  $p = 0.078$ ) (**Fig. 4E**), attributable to the lack of possible SNVs with appreciably positive allelic skew (no SNVs with allelic skew  $> 0.5$ ).

Analyzing all promoters (**Supplementary Dataset 8**), 27% had correlations between phyloP and allelic skew more extreme than *LDLR* (**Fig. 4F**; **Supplementary Fig. 25**). The most highly correlated example was the promoter of the broadly expressed *TNKS2*, which encodes a polymerase involved in various processes including Wnt/β-catenin signaling, telomere maintenance, and DNA repair<sup>121</sup> ( $\rho = -0.75$ ,  $p = 5.9 \times 10^{-26}$ ) (**Fig. 4G**). We found that 1.1% ( $n = 192$ ) of promoters exhibited significant correlation between allelic skew and phyloP for both LoF ( $\rho = -0.54$ ,  $p = 4.9 \times 10^{-11}$ ) and GoF ( $\rho = -0.67$ ,  $p = 4.7 \times 10^{-17}$ ) mutations, suggestive of stabilizing selection (**Fig. 4H**; **Supplementary Fig. 26**). Comparably, 42.5% ( $n = 7,645$ ) of promoters had a significant correlation only at LoF mutations, indicating the commonality of strong purifying selection against reductions in promoter activity (permutation  $p < 0.001$ ). Only 0.2% ( $n = 38$ ) of promoters showed significant correlation only at GoF mutations, including *EMILIN2* ( $\rho = 0.42$ ,  $p = 1.3 \times 10^{-6}$ ) (**Fig. 4I**), an extracellular matrix protein<sup>122</sup> (**Supplementary Fig. 27**). Genes with significant correlation between only negative allelic skew and phyloP had higher gene-level coding constraint ( $p < 0.001$ , Wilcoxon signed rank test) (**Supplementary Fig. 28**) and were enriched in pathways related to RNA or protein metabolism, cell cycle regulation, signal transduction, neurodevelopment, cellular stress responses, and embryonic lethality (**Supplementary Dataset 9**). MPAC thus enables improved regulatory variant interpretation and novel gene prioritization strategies, accounting for both the magnitude and direction of expression effects in the context of evolutionary constraint.

Constraint in promoter regions can reflect both *cis*-regulatory and downstream gene-level fitness effects. We explored this relationship using MPAC allelic skew to assign *cis*-regulatory impact,  $s_{het}$  to quantify gene-level loss-of-function (LoF) intolerance, and phyloP to measure constraint<sup>123–125</sup> (**Fig. 4J**). We observed that genes with higher  $s_{het}$  had higher correlations between allelic skew and phyloP (top 10%:  $\rho = 0.27$  vs. bottom 10%:  $\rho = 0.087$ ) (**Fig. 4K**), suggesting that the importance of the gene strongly influences the relationship between non-coding effect and constraint. For large negative skew mutations in promoters of the most LoF-intolerant genes, we observed extremely elevated constraint (phyloP = 6.52) (**Fig. 4J**). Expression-increasing mutations in the same promoters also showed significant, albeit reduced, levels of constraint (mean phyloP = 3.87). Surprisingly, large negative effect mutations for the most LoF-tolerant genes were highly constrained (bottom 10%: mean phyloP = 3.67) comparable to large positive effect functional mutations for the most LoF-intolerant genes (top 10%: mean phyloP = 3.88). Thus, even LoF-tolerant genes show constraint against likely deleterious promoter mutations, a finding consistent across alternative measures of gene constraint, including LOEUF, MOEUF, and AlphaMissense<sup>124,126</sup> (**Supplementary Fig. 29**).

## Discussion

Identifying causal non-coding variants is challenging due to the complex grammar underlying CRE function. MPRA have shown promise in quantitatively measuring non-coding variants' allelic skew to identify those likely underlying human traits and disease; however, oligo-based assays can only assay a maximum of a few hundred thousand variants at a time<sup>66</sup>. To address this, we developed MPAC, an extension of our previous Malinois model of MPRA activity<sup>78</sup>.

MPAC provides a scalable framework for genome-wide non-coding variant effect prediction, and requires significantly less training data than other models. We leveraged MPAC's scalability, which exceeds the largest empirical MPRAAs by orders of magnitude<sup>100</sup>, to predict non-coding functional effects of large databases of hundreds of millions of common, rare, *de novo*, and somatic variation genome-wide, and to perform deep mutational scans across all human promoters.

While we demonstrate MPAC's functional predictions reliably identify clinically relevant non-coding variation, there are important biological and methodological limitations to consider. First, our predictions are based on an episomal MPRA using a minimal promoter designed to interrogate CRE function that is best suited for a subset of non-coding sequences<sup>66,84</sup>. MPAC is unable to predict functional variants in UTRs, some repressors, large structural or copy number variants, or elements involved in chromatin looping. Reporter assays are also unable to link CREs with their target genes, requiring methods such as pooled CRISPRi screens or Hi-C to contextualize the effect of the CRE. From a modeling perspective, MPAC is trained on MPRA data from three cell lines which limits the TFs identified by the model to those expressed in K562, HepG2, and SK-N-SH. We also identify a small number of motifs that contribute to missed emVar predictions, which could be tested via an active learning approach to improve model accuracy. We expect that advances in pre-training, multi-modal model architectures, expanded MPRA training size<sup>76,85</sup> and cellular contexts will improve the model, especially improving cell type-specific, non-promoter regulatory elements.

As genomic medicine advances towards personalized treatments, the availability of predictions for every possible mutation across the human genome will have many applications. For example, deep mutational scanning has demonstrated clinical utility in coding regions<sup>127–131</sup>, yet the scale and complexity of the non-coding genome makes regulatory variant effect modeling difficult. We expect MPAC predictions to complement existing maps of CREs, providing nucleotide-level predictions for functionally informed GWAS fine-mapping and rare disease clinical interpretation<sup>111,132,133</sup>. We functionally nominate a set of 1,892 recurrent somatic, putatively tumorigenic mutations in COSMIC for deeper mechanistic study. Our comprehensive analysis of gnomAD SNVs quantifies the evolutionary forces acting on regulatory function and enables better clinical interpretation of rare, likely deleterious, non-coding variants. Lastly, our promoter saturation mutagenesis map facilitates exploration of the relationships between constraint and function at individual genes and equips disease researchers with functional predictions for all possible promoter SNVs. MPAC predictions are rapidly deployable on DNA sequence alone, ancestry-agnostic, independent of linkage disequilibrium, and do not rely on large sequencing cohorts to identify putatively causal functional variants.

Overall, the advent of highly performant non-coding variant effect predictors like MPAC to create prospective maps of variant function at the scale of the entire genome will enable genome interpretation to match the speed of variant discovery. As whole-genome sequencing becomes increasingly available across common, rare, and somatic disease cohorts, the need for effective variant interpretation will only grow. While MPAC focuses on non-coding regulatory variation, we expect its integration with advanced models of other genomic functions<sup>52,55,81,82,112,134–139</sup> will enhance personal genome interpretation and deepen our molecular understanding of human disease.

## *Data availability*

All MPAC predictions, additional datasets and model hyperparameters are available at Zenodo (<https://doi.org/10.5281/zenodo.15178434>) organized as follows:

Supplementary Dataset 1: MPAC\_UKBB\_BBJ\_GTEEx\_MPRA\_predictions  
Supplementary Dataset 2: MPAC\_DNase\_ASE\_predictions  
Supplementary Dataset 3: MPAC\_ClinVar\_predictions  
Supplementary Dataset 4: MPAC\_COSMIC\_predictions  
Supplementary Dataset 5: MPAC\_gnomAD\_predictions  
Supplementary Dataset 6: MPAC\_GWAS\_tag\_SNVs+LD\_predictions  
Supplementary Dataset 7: MPAC\_promoter\_saturation\_mutagenesis  
Supplementary Dataset 8: Promoter\_function\_constraint\_correlations  
Supplementary Dataset 9: Promoter\_gene\_set\_enrichment\_results  
Supplementary Dataset 10: MPAC\_model\_hyperparameters  
Supplementary Dataset 11: MPAC\_model\_artifacts

## *Code availability*

All code and documentation are deposited online as follows. Code used for MPAC variant effect predictions and to analyze and visualize data related to the benchmarking, ClinVar, and COSMIC work is available at <https://github.com/john-c-butts/MPAC/>. Code used to analyze and visualize data related to the gnomAD and promoter saturation mutagenesis work is available at [https://github.com/Reilly-Lab-Yale/MPAC\\_gnomAD\\_and\\_satmut](https://github.com/Reilly-Lab-Yale/MPAC_gnomAD_and_satmut).

## *Contributions*

J.C.B., S.R., S.J.G., R.I.C., R.T., and S.K.R. designed the study and contributed to the development of MPAC. J.C.B., S.R., S.J.G., M.N., K.A., R.G., R.I.C., R.T., and S.K.R. performed data analysis and interpreted results. J.C.B., S.R., M.N., R.T., and S.K.R. drafted the manuscript. P.C.S., R.T., and S.K.R. secured funding. R.T. and S.K.R. supervised the study. All the authors revised the manuscript and accepted its final version.

## *Competing Interests*

P.C.S. and R.T. have filed intellectual property related to MPRA. S.J.G., P.C.S., R.I.C., R.T., and S.K.R., have filed intellectual property related to MPRA models. P.C.S. is a co-founder and shareholder of Delve Bio, and was formerly a co-founder and shareholder of Sherlock Biosciences and Board Member and shareholder of Danaher Corporation. The other authors declare no competing interests.

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## Methods

### *Data preprocessing and model training*

We partitioned a previously generated MPRA dataset<sup>66</sup> into 11 roughly even sized splits based on the chromosome pairs from which the oligo sequences were derived. Pairs of source chromosomes were selected such that the sums of the chromosome identifiers were 23 (e.g., chr3 and chr20 form a pair). Next, we organized the data into 110 combinations of training, validation, and test sequences composed of 10, 1, and 1 pairs of source chromosomes, respectively. The training and validation groups were used to fit and select model parameters, respectively. Therefore, for every pair of source chromosomes, 10 models are fitted using unique training/validation combinations to use for benchmarking performance and making predictions. We then trained 110 models as previously described using these data set combinations using the code base available at <https://github.com/sjgosai/boda2> and hyperparameter settings defined in (**Supplementary Data 10**).

### *Predicting variant effects using MPAC*

Command line tools, the original Malinois model, and code base is available at (<https://github.com/sjgosai/boda2>). The 110 MPAC models are available at Zenodo (<https://doi.org/10.5281/zenodo.15178434>). Predictions are generated with the ‘vcf\_predict.py’ script as shown below:

```
python /opt/boda2/src/vcf_predict.py --artifact_path {10X $MODEL} --vcf_file ${VCF} --fasta_file ${FASTA} --output ${OUTPUT} --relative_start 9 --relative_end 181 --step_size 10 --strand_reduction mean --window_reduction mean
```

The above example generates 18 predictions per variant, tested in positions 9 to 181 (0-based, 10 bp intervals) which are reduced to a single reference and alternate activity ( $\log_2\text{FC}$ ) by averaging. Allelic skew is then calculated as the difference between alternate and reference activity. GRCh38\_no\_alt\_analysis\_set\_GCA\_000001405.15.fasta was downloaded from [https://www.encodeproject.org/files/GRCh38\\_no\\_alt\\_analysis\\_set\\_GCA\\_000001405.15/](https://www.encodeproject.org/files/GRCh38_no_alt_analysis_set_GCA_000001405.15/) and used as the reference genome for all analyses. All predictions were generated with the above prediction schema except for correlation with empirical MPRA activity (**Figure 1B**) which used the following:

```
python /opt/boda2/src/vcf_predict.py --artifact_path {10X $MODEL} --vcf_file ${VCF} --fasta_file ${FASTA} --output ${OUTPUT} --relative_start 99 --relative_end 100 --step_size 1 --strand_reduction mean --window_reduction mean
```

Which generates a single prediction with the variant centered at position 99 (0-based) to exactly reproduce the sequences tested by the empirical MPRA used for comparison<sup>66</sup>.

### *MPAC correlation with empirical MPRA*

Single, variant centered predictions were generated for all empirical MPRA sequences from<sup>66</sup> that passed QC, K562 (n=485,180), HepG2 (n=499,820), and SK-N-SH (n=485,034). Empirical  $\log_2\text{FC}$  was compared to predicted  $\log_2\text{FC}$  for computing Pearson’s correlation. Next, MPAC MPAC allelic skew and empirical Log2Skew were compared for empirically validated emVars ( $(\log_{10}\text{Padj}_\text{BF} \geq -\log_{10}(0.01) \& \text{abs}(\log_2\text{FC}) \geq 1) \& \text{Skew}_{\log_{10}\text{Padj}} \geq -\log_{10}(0.1)$ ) in K562 (n = 16,187), HepG2 (n = 14,029), and SK-N-SH (n = 14,723) from the same experimental dataset. Predictions and empirical measurements were correlated using Pearson’s method.

### *Correlation to DNase allele-specific effects variants*

DNase allele specific effects (ASE) data were obtained for the three MPAC cell types (K562, HepG2, and SK-N-SH) from Jeff Vierstra (unpublished). Significant ASE variants were called

using a minimum FDR threshold of  $\leq 0.05$ . As SK-N-SH samples had no significant hits they were dropped from subsequent analysis. ASE mean effect sizes were multiplied by -1 to match directionality of MPAC allelic skew (Alt – Ref). MPAC allelic skew was compared to DNase skew for all K562 and HepG2 ASE variants as well as the subset of MPAC emVars ( $|\text{allelic skew}| > 0.5$ ). Significant ASE variants were identified in previously performed MPRA experiments<sup>66</sup> and Log2Skew was compared to ASE effect size for both all ASE variants and those called emVars by MPRA ( $(\log_{10} \text{Padj\_BF} \geq -\log_{10}(0.01) \& \text{abs}(\log_{10} \text{FC}) \geq 1) \& \text{Skew\_logPadj} \geq -\log_{10}(0.1)$ ). All correlations were performed using Pearson's method.

For additional model comparisons precomputed Enformer 1KG predictions were downloaded from

(<https://console.cloud.google.com/storage/browser/dm-enformer/variant-scores/1000-genomes/enformer>) and filtered for significant ASE variants (HepG2  $n = 331$ , K562:  $n = 855$ ). All Pearson's correlations presented in **Fig. 1D** reflect this subset. Sei<sup>81</sup> predictions were generated for these same variants using the web interface (<https://hb.flatironinstitute.org/deepsea/>) and filtered for HepG2 and K562 DNase. For both Enformer and Sei the highest Pearson's correlation to empirical DNase was reported.

### *Correlation of Enformer and Sei DNase predictions to MPRA emVars*

All empirical emVars<sup>66</sup> present in the precomputed Enformer 1KG variants predictions (HepG2  $n = 10,407$ , K562 = 11,993) were downloaded from (<https://console.cloud.google.com/storage/browser/dm-enformer/variant-scores/1000-genomes/enformer>). Sei was downloaded and installed from (<https://github.com/FunctionLab/sei-framework>). Variant effect predictions for all emVars were generated using the following command 'sh 1\_variant\_effect\_prediction.sh ..vcfs2sei/emvar\_vcf.vcf hg38 ..sei\_preds/mpra\_emvars --cuda.' For both Enformer and Sei predictions all HepG2 and K562 DNase predictions were compared to MPRA skew and the highest Pearson's correlation was used.

### *Precision-Recall of high PIP GWAS variants*

We generated MPAC predictions for a set of statistically fine-mapped variants from the UK Biobank and Biobank Japan previously tested by empirical MPRA<sup>66</sup>. After filtering for autosomal variants tested in K562, HepG2, or SK-N-SH, the test set contained 1,869 variants: 934 positive (PIP > 0.9) and 935 negative (PIP < 0.01). MPAC predictions were reduced to a single score using the max(|allelic skew|) of K562, HepG2, or SK-N-SH. We also generated predictions for these variants with Sei<sup>81</sup> and LS-GKM<sup>86,140</sup>. Sei predictions were generated with the following command 'sh 1\_variant\_effect\_prediction.sh ..vcfs2sei/traits\_prc\_hg38\_vars\_for\_SEI\_preds.vcf hg38 ..sei\_preds/ukbb\_gtex --cuda' and the max(|allelic skew|) prediction for DNase in all cell types was used. LS-GKM and model weights were downloaded from (<https://www.beerlab.org/deltasvm/>). HepG2 predictions were generated using the following command 'perl deltasvm.pl ref\_fasta alt\_fasta SupplementaryTable\_hepg2weights.txt hepg2\_out' and K562 predictions with 'perl deltasvm.pl ref\_fasta alt\_fasta SupplementaryTable\_k562weights.txt k562\_out'. The max(|allelic skew|) prediction from K562 and HepG2 was used. Precision and recall curves, as well as AUPRC measurements were generated using scikit-learn 1.4.1.post1<sup>141</sup>.

### *Precision-Recall of high PIP GTEx variants*

MPAC predictions were generated for a set of previously described high (> 0.9) and low (< 0.01) PIP eQTLs<sup>66</sup>. After filtering for autosomal variants empirically tested in K562, HepG2, and SK-N-SH, SNVs the test set contained 22,720 variants: 11,354 positive and 11,366 negative.

MPAC predictions were reduced to a single score using the max(|allelic skew|) of K562, HepG2, and SK-N-SH. Sei<sup>81</sup> predictions were generated with 'sh 1\_variant\_effect\_prediction.sh {./vcfs2sei/gtex\_prc\_hg38\_vars\_for\_SEI\_preds.vcf} {hg38} {./sei\_preds/ukbb\_gtex} --cuda' and the max(|allelic skew|) prediction for DNase in all cell types was used. LS-GKM<sup>86,140</sup> predictions were generated using the command 'perl deltasvm.pl {ref\_fasta} {alt\_fasta} {SupplementaryTable\_hepg2weights.txt} {hepg2\_out}' for HepG2 and 'perl deltasvm.pl {ref\_fasta} {alt\_fasta} {SupplementaryTable\_k562weights.txt} {k562\_out}' for K562. The max(|allelic skew|) prediction from K562 and HepG2 was used. As before LS-GKM and model weights were downloaded from (<https://www.beerlab.org/deltasvm/>). Precision and recall curves, and AUPRC measurements were generated using scikit-learn 1.4.1.post1<sup>141</sup>.

### Missed MPAC prediction analysis

To understand sources of MPAC missed predictions compared to empirical MPRA, we subset all MPRA emVars for those with an |allelic skew| > 0.5 and an MPAC predicted |allelic skew| < 0.05. These incorrectly predicted emVars were independently identified from each cell type, split into 80/20 training and test sets and matched with a randomly sampled set of correctly predicted sequences. An LS-GKM model<sup>86,140</sup> was trained for each cell type using the command 'gkmtrain {positive\_train\_fasta} {negative\_train\_fasta} {output}'.

Using these models, importance scores were generated using gkmexplain<sup>88</sup> and passed to tfmodisco-lite (<https://github.com/jmschrei/tfmodisco-lite>)<sup>87</sup> for de-novo motif identification. tfmodisco-lite motifs were matched to known TF motifs using TOMTOM (MEME version 5.5.7, HOCOMOCOv11\_core\_HUMAN)<sup>142</sup>. We next used FIMO<sup>143</sup> ('fimo --no-pgc -o {outfile} {modisc\_pwms} {emvar\_fasta}') to quantify the presence of motifs identified by tfmodisco-lite in correctly predicted emVar sequences. FIMO hits were considered if they overlapped the variant (oligo position 100) and had a q-value < 0.01. Lastly, to determine abundance of de-novo motifs in the correctly predicted emVars the total number of significant FIMO hits with a significant TOMTOM match (q-value < 0.05) to a known motif was divided by the total number of correctly predicted emVar sequences.

### ClinVar analysis

The ClinVar VCF was downloaded from ([https://ftp.ncbi.nlm.nih.gov/pub/clinvar/vcf\\_GRCh38/archive\\_2.0/2023/clinvar\\_20230930.vcf.gz](https://ftp.ncbi.nlm.nih.gov/pub/clinvar/vcf_GRCh38/archive_2.0/2023/clinvar_20230930.vcf.gz), fileDate: 2023-09-30) and MPAC predictions were generated for all ClinVar variants prior to filtering for non-coding variants. To focus on non-coding SNVs, we filtered transcripts from GENCODE v44 basic annotations<sup>144</sup> (downloaded from [https://www.gencodegenes.org/human/release\\_44.html](https://www.gencodegenes.org/human/release_44.html)) that were located on chr1-22, had a transcript\_type column = "protein\_coding", a non-missing hgnc\_id, and tag column matching "Ensembl\_canonical" but not matching "readthrough\_gene". We then removed variants intersecting any exons. To remove potential splice disrupting variations, we also removed SNVs in intronic regions up to -20 from 3' splice sites or +6 from 5' splice sites, consistent with splice region definitions from MaxEntScan<sup>144,145</sup>. After filtering a total of 180,032 variants classified as pathogenic (ClinVar 'Pathogenic' or 'Likely\_pathogenic') or benign (ClinVar 'Benign' or 'Likely\_benign') were used for all downstream analyses. ClinVar variants were intersected with peaks of DNase I Hypersensitivity (DHS) downloaded from (<https://www.encodeproject.org/files/ENCFF503GCK/>)<sup>146</sup> and promoters. Promoters were defined as 250 bp immediately upstream of the TSS of filtered GENCODE transcripts. emVar proportions for pathogenic and benign variants are calculated as the number of emVars labeled non-DHS, DHS, or Promoter over the total number of variants in each category. Significance was tested by the Chi-Square Test. Enrichments for pathogenic and benign variants were

calculated by odds ratio of emVars in each respective group and tested by Fisher's exact test to calculate *p* values.

Sei<sup>81</sup> predictions were generated for all non-coding SNVs using the command 'sh 1\_variant\_effect\_prediction.sh {clinvar\_exon\_filtered\_vars.vcf} {hg38} {/clinvar\_preds} --cuda' and filtered for K562, HepG2, and SK-N-SH ENCODE DHS predictions. For a given category (All ClinVar, DHS, Promoter) all pathogenic variants were compared to an equal random sample of benign variants 100 times. For both MPAC and Sei predictions the max(|allelic skew|) prediction from K562, HepG2, and SK-N-SH was used in precision recall calculations. Precision and recall curves, and AUPRC measures were generated using scikit-learn 1.4.1.post1<sup>141</sup>.

### COSMIC analysis

MPAC predictions were generated for all non-coding COSMIC<sup>23</sup> (v98, downloaded from (<https://cancer.sanger.ac.uk/cosmic/download/cosmic/v98/noncodingvariantsvcf>)). Variants were filtered for only SNVs derived from whole genome sequencing ('Whole\_Genome\_Reseq' == 'y' and 'Whole\_Exome' == 'n') from (<https://cancer.sanger.ac.uk/cosmic/download/cosmic/v98/noncodingvariantstsv>). Variant recurrence was calculated as the number of COSMIC mutation IDs ('GENOMIC\_MUTATION\_ID') present after filtering. MPAC predictions were generated for ETS-TF disrupting putative drivers previously tested by MPRA<sup>98</sup>. We calculated percent emVar recovery as the number of MPAC emVars divided by empirically defined emVars.

Variants were subset into recurrent (*n* ≥ 2) or unique (*n* = 1) by ID count and feature annotations, promoters (as defined in ClinVar analysis), cancer promoters (CNC)<sup>99</sup>, recurrent promoters, and recurrent CNC promoters were assigned by intersection. CNC promoters were downloaded from (<https://cnccdatabase.med.cornell.edu/>) with the query 'promoters' and using all unique gene names for the final list. Variants were categorized into putative regulatory elements by intersection with DHS elements from (<https://www.encodeproject.org/files/ENCFF503GCK/>)<sup>146</sup>. Odds ratios (OR) were calculated for the proportion of emVars in the category of interest versus the remainder of COSMIC variants. For allelic skew bin comparisons bins were defined as the mean allelic skew of all predictions. ORs were calculated for promoter variants in the allelic skew bin versus distal variants. For activity comparisons variants were binned by the max(ref | alt) log<sub>2</sub>FC and the OR that a recurrent or unique variant was an emVar was calculated. For all ORs Fisher's exact test was used to calculate *p* values and 95% confidence intervals.

### MPAC gnomAD SNV predictions and analysis

We used MPAC to predict variant effects for all 646,033,065 SNVs reported in 76,156 whole genomes analyzed by gnomAD v3.1.2 on hg38<sup>29</sup> (downloaded from <https://gnomad.broadinstitute.org/downloads#v3-variants>). We filtered to include SNVs that: 1) passed gnomAD QC, 2) were located on autosomes (chr1-22), 3) had non-zero minor allele frequency (MAF = min(AF, 1 - AF) > 0), 4) non-zero allele count (AC > 0), and 5) total number of alleles observed is at least half of gnomAD (AN ≥ 76,156).

We annotated SNVs by 1) overlapping Zoonomia 241-way base-level phyloP scores<sup>26</sup> (downloaded from [https://cgl.gi.ucsc.edu/data/cactus/241-mammalian-2020v2-hub/Homo\\_sapiens/241-mammalian-2020v2.bigWig](https://cgl.gi.ucsc.edu/data/cactus/241-mammalian-2020v2-hub/Homo_sapiens/241-mammalian-2020v2.bigWig)), and 2) annotations of mutation rate from Roulette predictions<sup>147</sup> (as annotated in CADD v1.6<sup>111</sup> downloaded from

[https://krishna.gs.washington.edu/download/CADD/v1.6/GRCh38/whole\\_genome\\_SNVs.tsv.gz](https://krishna.gs.washington.edu/download/CADD/v1.6/GRCh38/whole_genome_SNVs.tsv.gz)). We removed variants without a phyloP annotation (1.7% of variants).

To focus on non-coding SNVs, we removed variants intersecting our previously defined exons and splice regions on filtered GENCODE transcripts (see “*ClinVar analysis*”). This resulted in a final set of 513,886,257 SNVs for downstream analyses.

GWAS Catalog (accessed 2024-12-19)<sup>10</sup> SNVs were expanded to include all variants in high LD ( $r^2 \geq 0.7$ ) in each of three superpopulations (ASN, AFR, EUR). This set was assigned MPAC predictions by subsetting our filtered GWAS prediction set to those with a MAF  $\geq 1\%$ , then joining by rsID.

#### *gnomAD SNV functional annotations*

We categorized non-coding SNVs by intersecting with the ENCODE cCRE class annotations<sup>37,144,145</sup> (downloaded from <https://downloads.wenglab.org/Registry-V4/GRCh38-cCREs.bed>), specifically promoter-like signatures (PLS), proximal enhancer-like signatures (pELS), and distal enhancer-like signatures (dELS). SNVs not falling into the above categories were further classified as “Other cCREs” if they overlapped any remaining cCRE annotations, or labeled as “non-cCRE” if overlapping no annotations.

For the TF binding enrichment analysis, we labeled SNVs as overlapping DNase I TF footprints if they intersected at least one consensus footprint from Vierstra et al.<sup>104</sup> (downloaded from <https://zenodo.org/records/3905306>) and overlapping TF ChIP-seq peaks if they intersected at least one peak from ChIP-Atlas<sup>105,106</sup>. Odds ratios (OR) were calculated using Fisher’s exact test with a +1 pseudocount correction to calculate the OR, p-value, and 95% CI.

To compare mean phyloP between non-coding and coding functional variants, we annotated SNVs by Ensembl VEP<sup>105,148</sup> based on gnomAD v3.1.2 annotations using the worst predicted consequence as reported in the sequence ontology ranking provided in the Ensembl VEP documentation ([https://ensembl.org/info/genome/variation/prediction/predicted\\_data.html](https://ensembl.org/info/genome/variation/prediction/predicted_data.html) retrieved 2024-06-10) prior to removing SNVs overlapping exons or splice regions.

#### *gnomAD evolutionary constraint analyses*

We binned SNVs by their MPAC predicted mean log<sub>2</sub>FC in activity across all cell lines or mean log<sub>2</sub>FC allelic skew. We used the enrichment of rare (AF < 0.1%) vs. common (AF  $\geq 0.1\%$ ) SNVs as a measure of recent purifying selection and compared across MPAC allelic-skew bin for each ENCODE cCRE class. We omitted bins with less than 50 variants. We calculated the odds ratios (OR) based on counts of rare vs. common SNVs belonging to a focal subset of SNVs (MPAC allelic-skew bin x ENCODE cCRE class) relative to that of all other SNVs in the genome, using Fisher’s exact test with a +1 pseudocount correction to calculate the OR, p-value, and 95% CI.

#### *MPAC promoter saturation mutagenesis predictions*

Starting from the filtered list of 18,761 GENCODE transcripts (see “*ClinVar analysis*”), we used MPAC to conduct *in silico* saturation mutagenesis of all 3,000 possible single nucleotide mutations across the 1 kb promoter regions located upstream of the TSS. We masked mutations intersecting any exons or splice regions of filtered GENCODE transcripts (see “*ClinVar analysis*”), resulting in 18,658 promoter regions with any mutations after masking. At each base position, the mean activity (log<sub>2</sub>FC) or allelic skew (log<sub>2</sub>FC) is that of all three non-reference mutations averaged across all three cell lines (nine values total). The 1 kb

promoter regions were used for **Supplementary Fig. 21** and **22**. All subsequent analyses were conducted on the 250 bp promoter region upstream of the TSS. Means at each distance from the TSS were calculated using promoters that had unmasked values at that specific position.

### *Motif analysis of promoter regions*

DNA sequences for 250 bp promoter regions were scanned for TF binding motifs using FIMO from the memes R package<sup>143,149</sup> and position weight matrices from JASPAR 2024<sup>150</sup> with accessions: TATA (MA0108.3), SP1 (MA0079.5), NRF1 (MA0506.1), ELK1 (MA0028.2), NFYC (MA1644.1), CREB1 (MA0018.3), YY1 (MA0095.2), CTCF (MA0139.1), and ZNF143 (MA0088.2). Significant motifs were called at default  $p < 4 \times 10^{-4}$ , which corresponds to rescaling the default FIMO value of  $1 \times 10^{-4}$  to 250 bp promoters as specified in <https://meme-suite.org/meme/doc/fimo-tutorial.html>. Motif count profiles were based on the positions of motif midpoints relative to the TSS.

### *Gene-level expression annotations*

Gene expression in transcripts per million (TPM) were downloaded from the Human Protein Atlas for K562, HepG2, and SK-N-SH cell lines<sup>151</sup>. Using Pearson's  $r$ , the  $\log_{10}$ TPMs were then correlated against the mean MPAC-predicted activity of each 250 bp promoter region, computed as the mean of activities centered on every position in the region. Gene expression annotations were joined to filtered GENCODE transcripts (see “*ClinVar analysis*”) by matching Ensembl gene IDs, Ensembl transcript IDs, or gene names.

### *Gene-level coding constraint stratification*

Promoters were annotated by four different scores of gene-level coding constraint: GeneBayes  $s_{het}$  LoF-intolerance<sup>125</sup>, LOEUF LoF-intolerance and MOEUF missense-intolerance from gnomAD v4.0<sup>124</sup>, and mean AlphaMissense missense pathogenicity<sup>126</sup>. Each of these were binned into deciles for the coding constraint stratification analyses. Gene-level coding constraint annotations were joined to filtered GENCODE transcripts (see “*ClinVar analysis*”) by matching Ensembl gene IDs, Ensembl transcript IDs, or gene names.

### *Promoter correlations between function and constraint*

For each promoter, we calculated Spearman correlation ( $\rho$ ) between MPAC-predicted allelic skew at each position (mean  $\log_2$ FC of the three possible mutations across three cell lines) and across-species base-level constraint (max(0, phyloP) scores). We performed this calculation separately for negative or positive allelic-skew variants, requiring 50 unmasked positions that had both a phyloP score and a non-zero skew prediction. Among analyzed promoters, 17,392 had defined correlations for at least one category (positive or negative allelic-skew variants), while 16,995 had both. We identified promoters with significant correlations by comparing them to a permutation-based null distribution created by shuffling base positions among 250 bp promoter regions, where we defined promoters as significant if their correlation values fell below the bottom 0.01% (for negative allelic-skew) or above the top 0.01% (for positive allelic-skew) of this null distribution.

Gene set enrichment analyses were conducted using the Enrichr web server<sup>152</sup> with the negative allelic-skew correlation, positive allelic-skew correlation, or both correlation gene sets using the background set of 17,392 genes where either the positive or negative allelic-skew correlations was well-defined, focusing on Reactome Pathways 2024, GO Biological Process 2025, and MGI Mammalian Phenotype Level 4 gene sets with  $FDR < 0.05$ .

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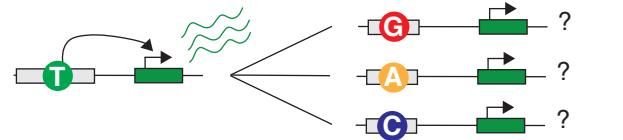
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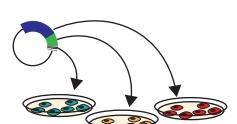
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**A**

The impact of non-coding variation on CRE function is unclear without validation

### Empirical MPRA

Choose ~10-100K variants for Library

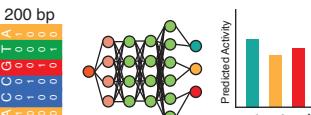


Quantification of Oligo Activity

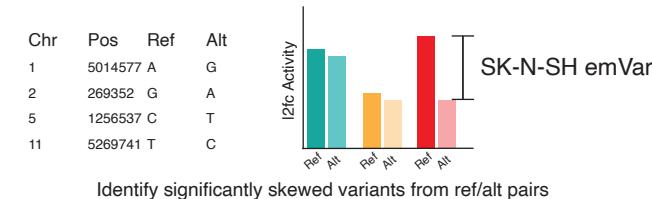
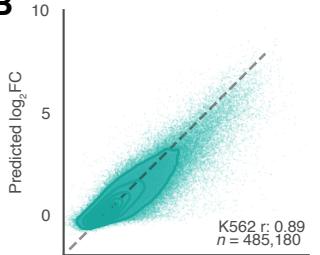
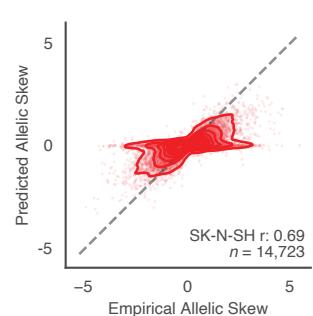
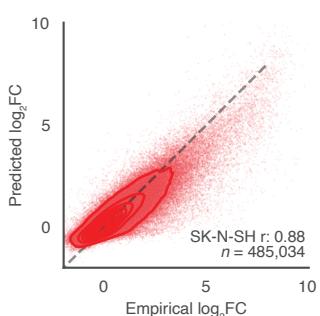
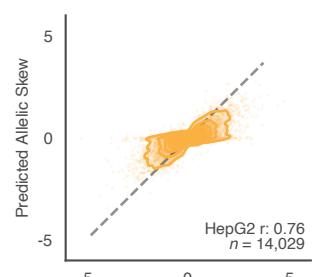
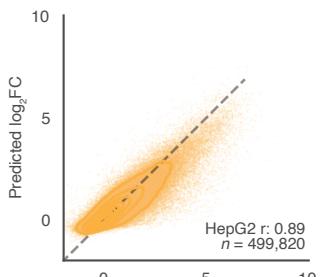
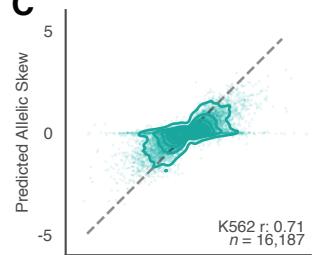
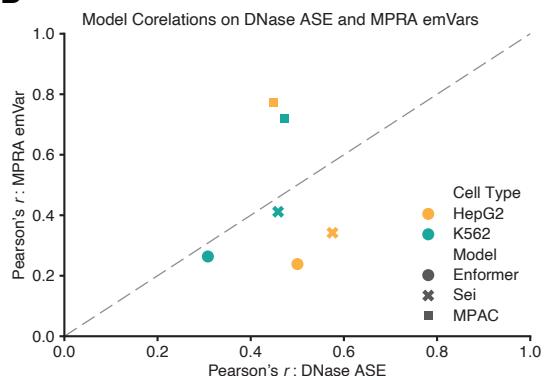
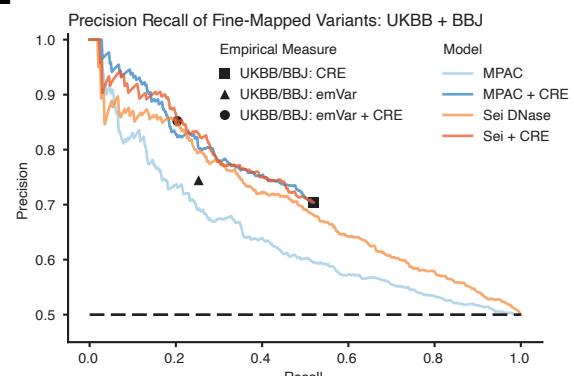


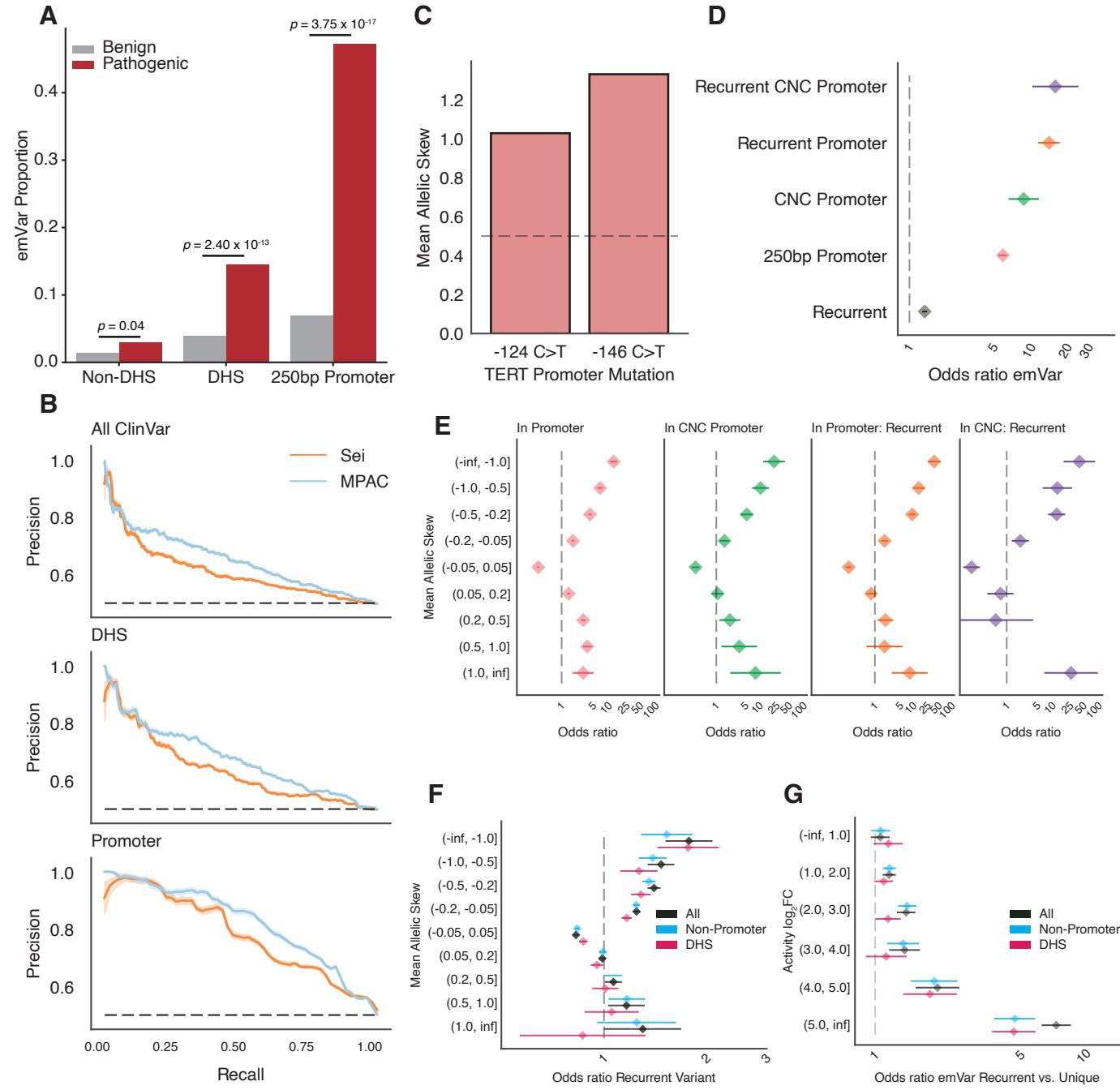
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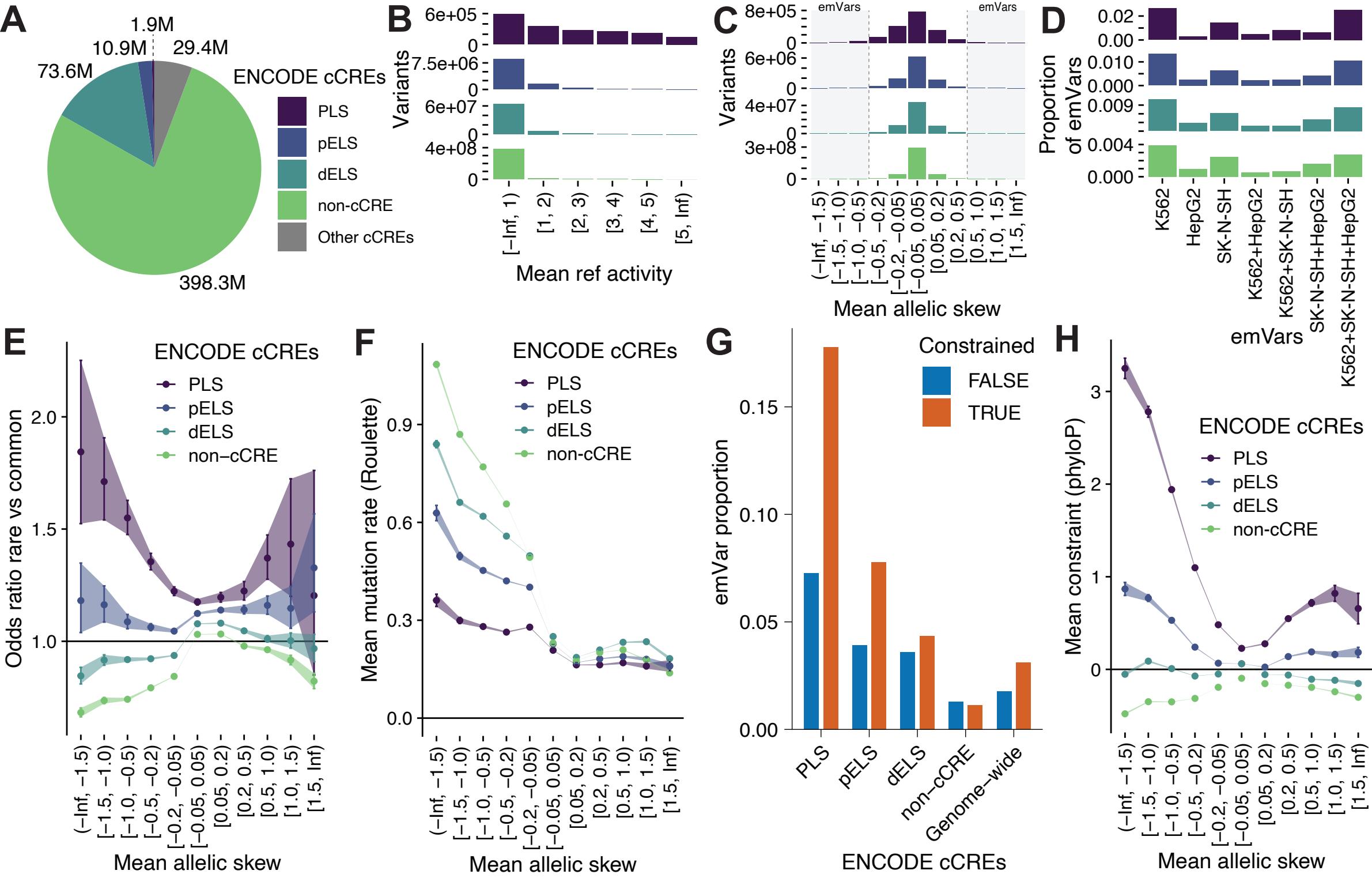
Test all relevant variants

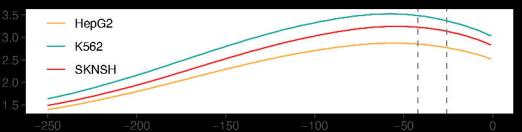


Model Prediction of MPRA Activity

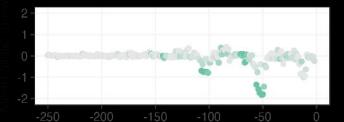
**B****C****D****E**







LDLR (chr10:11089143-11089213)



TNKS2 (chr10:91798426-91798776)

