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Predicting genetic variants effect on genomic Regulatory Elements and CRISPR off-targets

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Introduction

Transcription Factors

Transcription Factors (TFs) (**Fig.2.1**) are fundamental regulatory proteins playing a key role in regulating the transcriptional state, differentiation and developmental state of cells (Lambert *et al.*, 2018; Reimold *et al.*, 2001; Whyte *et al.*, 2013). TFs present a modular structure, divide in three domains (Latchman, 1997): (i) the DNA binding domain, (ii) the activation domain, and (iii) the signal sensing domain. The DNA binding domain guides the TF to its target sites on the genome. The activation domain interacts with other transcriptional regulators. The signal sensing domain captures external signals, transmitting them to the whole transcriptional complex. In human, about 1600 proteins are currently thought to function as TFs (Babu *et al.*, 2004). Therefore, approximately the 8% of human genes encode for TFs. Transcription Factors often exploit their regulatory function on genes, by acting in a coordinate fashion. Moreover, TFs regulate multiple genes in different cell-types (Lambert *et al.*, 2018). TFs perform their regulatory function by employing different strategies: (i) helping or blocking RNA polymerase binding (Fuda *et al.*, 2009), (ii) weakening the DNA-histones associations opening the chromatin, (iii) catalyzing histones deacetylation (Liu *et al.*, 2016), or (iv) strengthening the DNA-histone complexes closing the chromatin. By binding short DNA sequences (7-20 nucleotides (Stewart *et al.*, 2012)) called Transcription Factor binding sites (TFBS), TFs finely regulate gene expression in a cell-specific manner. TFBS are located within gene promoters (Whitfield *et al.*, 2012) or in distal regulatory elements, such as enhancers, silencers or insulators (Gotea *et al.*, 2010; Lemon and Tjian, 2000; Nolis *et al.*, 2009). Although TFBS show recurring sequence patterns, which are often referred to as *motifs*, TFs bind similar but not identical sequences, that can differ in a few nucleotides. The precise identity and configuration of TFBS, together with the conformation of flanking chromatin regions, critically regulate TFs regulatory function of cells (Mendenhall *et al.*, 2013; Maurano *et al.*, 2015). During DNA binding, Transcription Factors use a combination of electrostatic and Van der Waals forces. Although TFs bind their target sequences with high specificity, not all nucleotides in the binding sites directly interact with the TF. Since some interactions between the TF and the TFBS nucleotides are weaker than others, TFs do not bind a single signal sequence, but a subset of a closely related targets. However, the sequence composition defines the TF-DNA binding strength (binding affinity). Several studies linked different diseases and cancer types to genetic variants occurring in TFBS (Docquier *et al.*, 2005; Katainen *et al.*, 2015; Yu *et al.*, 2019). Moreover, the misregulation of gene expression governed by TFs caused by variants occurring in TFBS could affect the entire cell environment and be propagated to neighboring cells. Therefore, identifying such regulatory motifs would provide fundamental insights on the complex mechanisms governing gene expression and the cell environment.

2.1 Discovering Transcription Factor Binding Site motifs

Several experimental assays have been developed to determine the binding site sequences of TFs in living cells or organisms (*in vivo*), or in test-tubes using synthetic or purified components (*in vitro*) (Jolma and Taipale, 2011). Early methods, like electrophoretic mobility shift assay (EMSA) (Garner and Revzin, 1981) or footprinting (Hampshire *et al.*, 2007), generally search the binding sites for the investigated TFs analyzing a relatively small number of target sequences, producing small datasets of bound sequences. The introduction of *in vitro* and *in vivo* high-throughput protocols, like PBM, SELEX or ChIP methods (Berger *et al.*, 2006; Jolma *et al.*, 2010; Collas and Dahl, 2008), enabled the analysis of most of all possible target sites for the investigated factors, returning large datasets of bound sequences, and providing an unprecedented opportunity to study and determine TFs binding landscapes. Experimental assays can recover the bound sequences along with their binding affinity values. However, such datasets can hide unbound sequences erroneously reported as binding sites (false positives). Moreover, most assays capture several additional nucleotides the target sites, limiting the data resolution, and making manual analyses difficult and often unfeasible. Motif discovery algorithms provide a computational framework



Figure 2.1. A human transcription factor (CTCF) binding its DNA target sequence.

to analyze the datasets produced by experimental assays, discovering the sequences potentially bound by TFs and reporting their predicted affinities. Given a sequence dataset, motif discovery algorithms recover sets of short and similar sequence elements. The prioritized sequence elements are later used to construct a motif model, summarizing the diverse binding site configurations observed among the prioritized sequences, and encoding their recurrent patterns and similarities. The development of motif discovery methods is one of the most investigated and active research fields in computational biology (Pavesi *et al.*, 2004a; Tompa *et al.*, 2005; D'haeseleer, 2006; Das and Dai, 2007; Zambelli *et al.*, 2013). Several different algorithms and models have been proposed to discover and represent TFBS motifs. Position weight matrices (PWMs) (Stormo, 2000) are the most popular models to represent TFBS motifs. PWMs are powerful and interpretable models, encoding the probability of observing a given nucleotide in each TFBS position. However, PWMs have some limitations, like the assumption of independence among the binding site positions. Therefore, several alternative models have been proposed to describe TFBS motifs (Siddharthan, 2010; Gorkin *et al.*, 2012; He *et al.*, 2021). The derived motif models can be employed in many downstream analyses, like searching potential binding site occurrences in regulatory genomic sequences, predicting the sets of genes regulated by the investigated TFs, or assessing how genetic variants could affect the factors' binding landscape.

2.1.1 Experimental methods to discover Transcription Factor Binding Sites

During the last decades, several techniques have been introduced to experimentally identify and assess TF binding sites and binding preferences (Jolma and Taipale, 2011) (Fig.2.2 (A) and Table 2.1). Early studies on TF binding focused their analysis on gene promoters (Stormo, 2000) and employed in vitro methods, such as Electro-Mobility Shift Assay (EMSA) (Garner and Revzin, 1981) or DNase footprinting (Galas and Schmitz, 1978). EMSA exploits non-denatured polyacrylamide gel properties to separate bound and unbound DNA sequences, while DNase footprinting combines EMSA with DNase I cleavage. Generally, these assays produce datasets of a few hundreds of bound sequences, exploring a limited spectrum of TFs binding landscape. Moreover, EMSA and DNase footprinting often suffer

Experimental assay	Description	Output	<i>De novo</i> motif discovery capability	Type	Identification of genomic binding locations	Throughput
Competition EMSA	Bound DNA sequences are identified by observing changes in the electrophoretic migration of DNA sequences through non-denatured polyacrylamide gel.	Bound DNA sequences.	No. Used to validate known binding sites.	<i>in vitro</i>	No	Low
DNase footprinting	Pools of DNA sequences are incubated with the TF of interest; then, the DNA is degraded using DNase-I. The unbound fragments are cut in all positions, while the bound DNA is protected by the TF.	Bound DNA sequences.	No. Used to validate known binding sites.	<i>in vitro</i>	No	Low
Protein Binding Microarrays	Arrays of 40,000 spots with short immobilized DNA sequences are incubated with a GST-tagged TF, and then washed to remove weakly bound proteins. The bound sequences are identified through fluorescence-based detection.	Continuous values describing fluorescence intensity on each array spot.	Yes. Limited to short motifs (12bp).	<i>in vitro</i>	No	High
HT-SELEX	The TF is added to a pool of randomized DNA fragments. The bound sequences are selected and constitute the starting pool for the next experimental round. The procedure is repeated for several rounds. Sequencing is employed to recover the sequence of the bound DNA fragments.	DNA sequences.	Yes	<i>in vitro</i>	No	High
ChIP-based technologies	TF-DNA complexes are crosslinked with formaldehyde, and immunoprecipitated employing TF-specific antibodies. The bound sequences are then prioritized employing qPCR microarrays (ChIP on Chip) or through sequencing (ChIP-seq). ChIP-exo integrates exonuclease treatment to enhance sequence resolution.	Genomic binding location coordinates.	Yes. Limited by the inability to distinguish direct and indirect binding.	<i>in vivo</i>	Yes	Low

Table 2.1. In vivo and in vitro experimental assays to identify and validate Transcription Factor binding sites. Generally, EMSA and DNase footprinting are used to validate known TFBS, while currently PBM, HT-SELEX, and ChIP-based methods are preferred to discover novel binding sites. Importantly, ChIP-based assays are the only methods that recover the TF genomic binding locations. The throughput column refers to the number of samples that can be processed in parallel by each method (high: about hundreds of samples; low: few samples).

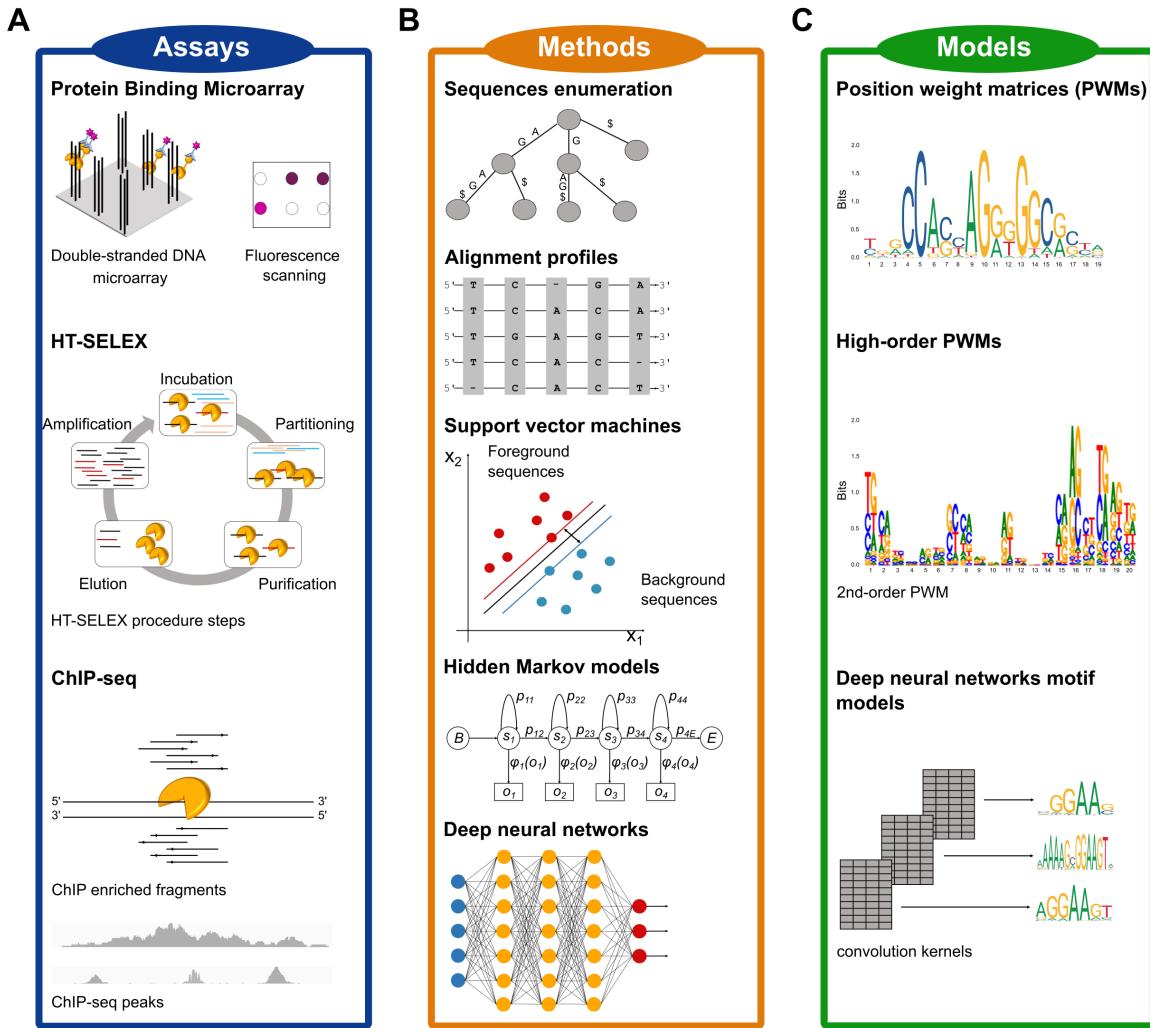


Figure 2.2. Experimental and computational methods to discover TFBS and popular models to represent binding site motifs. (A) Protein binding microarray (PBM), HT-SELEX and ChIP-seq during the last years have become the most popular assays to determine TF binding profiles and identify their target sites (TFBS). (B) Computational motif discovery methods can be grouped in five classes, based on the algorithms employed to discover TFBS: enumerative, alignment-based, probabilistic graphical models-based, support vector machines (SVMs), and deep neural network (DNN) based methods. (C) TFBS sequences prioritized by motif discovery algorithms are encoded in computational models representing the binding preferences of the investigated TFs.

from high false positive rates due to the identification of unwanted protein-DNA interactions, caused by nonspecific DNA binding factors potentially leading to wrongly measured binding preferences (Jolma and Taipale, 2011). The introduction of NGS technologies revolutionized the study of TFBS identification by encouraging researchers to develop methods that exploit the power of massively parallel sequencing, to identify TFBS. These methods have two major advantages: (i) they do not require any prior knowledge on the binding site sequence (Jolma and Taipale, 2011; Zia and Moses, 2012), and (ii) produce datasets of thousands of bound sequences allowing to better characterize TFs binding preferences (Stormo and Zhao, 2010). Protein binding microarrays (PBMs) (Berger *et al.*, 2006; Berger and Bulyk, 2009) recover short TFBS sequences (10 bp), and measure TF binding preferences *in vitro*. In PBMs a tagged TF is released on a glass slide containing thousands of spots filled with short immobilized DNA sequences. The tagged TFs are then incubated with fluorescent antibodies against the tag and subsequently washed to remove weakly bound factors. The fluorescence and DNA sequence enrichment are then used to quantify the TF-DNA binding strength and capture the bound sequences. Generally, the recovered sequences do not contain nucleotides flanking the investigated binding sites, producing high resolution datasets. However, since the number of possible sequences grows as a function of the target length, PBMs can assess only a limited number of target sequences (Jolma and Taipale, 2011; Zia and Moses, 2012). Therefore, PBMs analysis is usually constrained to binding sites 10-12 bp long. HT-SELEX (Jolma and Taipale, 2011; Jolma *et al.*, 2010) is a widely used *in vitro* method, coupling SELEX with high throughput sequencing.

A TF is released on a pool of randomized DNA sequences, to allow the factor to select its target sites. The resulting TF-DNA complexes are separated from unbound sequences, and subsequently amplified through PCR and sequenced. The resulting DNA library is enriched in binding sites for the studied TF and is used as the starting pool for another SELEX run (Jolma and Taipale, 2011; Jolma *et al.*, 2010). SELEX captures the binding preferences of TFs without requiring any prior knowledge on their target sites (Collas and Dahl, 2008). Since SELEX reaction is typically performed in liquid phase and consequently does not suffer from physical constraints, the sequence space covered by HT-SELEX is often larger than that of PBM. Moreover, by coupling sequencing with DNA barcode indexing, HT-SELEX allows to analyze of hundreds of TFs in a single run. HT-SELEX produces datasets of thousands of high resolution bound sequences, which include only a few nucleotides flanking the binding sites. However, since the starting DNA library is constituted by randomized sequences, HT-SELEX cannot recover the genomic binding locations for the investigated factor. The introduction of chromatin immunoprecipitation (ChIP) technologies (Collas and Dahl, 2008) radically changed the study of TFBS binding, enabling the genome-wide identification of regions bound by TFs *in vivo*. In ChIP the TF-DNA complexes are cross-linked using formaldehyde. The DNA is then fragmented in 100-1000 bp long fragments, and subsequently immunoprecipitated with antibodies specific for the investigated TF. To recover the bound sequences, the cross-links are reverted. Then, the resulting fragments are amplified through microarray hybridization (ChIP on Chip (Collas and Dahl, 2008; Pillai and Chellappan, 2015)), or sequencing (ChIP-seq (Johnson *et al.*, 2007; Mardis, 2007)). To locate the binding regions, the recovered DNA fragments are mapped onto the genome. After ChIP-seq reads mapping, peak calling algorithms (Thomas *et al.*, 2017; Guo *et al.*, 2012; Zhang *et al.*, 2008) are employed to predict the genomic binding locations for the investigated factor. Peak calling algorithms identify the genomic regions showing greater enrichment in mapped DNA probes with respect to a control experiment (ChIP-seq peaks), and mark those regions as binding locations, or peaks (Pepke *et al.*, 2009). ChIP methods produce large datasets of thousands of genomic regions enriched in TFBS, whose length ranges from few hundreds to thousands of nucleotides. Although ChIP technologies, and particularly ChIP-seq, are currently considered the current 'golden standard', they have several limitations. (i) experimental results are influenced by chromatin states (most TFs bind only open chromatin [1]). (ii) ChIP can detect indirect binding, identifying other TFBS not belonging to the investigated factor (Worsley Hunt and Wasserman, 2014). (iii) ChIP-seq peaks may be false positives, recovered because of poor antibody quality, for example (Pickrell *et al.*, 2011). (iv) ChIP-seq returns low resolution datasets, whose sequences include several nucleotides flanking the target TFBS. ChIP-exo (Rhee and Pugh, 2011) addresses the latter issue, employing a lambda exonuclease to trim ChIP sequences, removing some of the nucleotides flanking the target sites. In summary, the current high-throughput assays produce large datasets consisting of thousands of sequences potentially containing all possible binding configurations of TFBS allowing a better characterization of TFs binding landscapes. However, the sequences recovered by most of the described methods contain nucleotides flanking the TFBS, and only PBM and HT-SELEX data marginally suffer marginally from this issue. Therefore, computational methods and models to identify and characterize TFBS become fundamental to exploit the information contained in the datasets produced by experimental assays.

2.1.2 Computational methods to discover Transcription Factor Binding Sites

TFBS motif discovery is one of the most investigated problems in computational biology (Tognon *et al.*, 2022). During the last decades, several different algorithmic approaches have been proposed to discover potential binding sites in DNA sequences: *enumerative*, *alignment-based*, *probabilistic graphical models*, *support vector machines-based*, and *deep neural network-based* methods (**Fig.2.2 (B)**). Regardless of the algorithms employed and the computational model used to represent TFBS motifs, the TFBS motif discovery problem can be formalized as follows. Given a set of positive DNA sequences S , obtained from an experimental assay targeting a certain TF, and a set of negative sequences B , the goal is to find one or more recurrent, short and similar subsequences in S , that maximize the discriminatory power between S and B . Such subsequences are called patterns or motifs, and are likely bound by the investigated TF. The negative set B can contain randomly generated or selected genomic sequences, with similar nucleotide content and length of those in S . The retrieved patterns are used to construct and train a computational model M (motif model), representing the discovered motif. These models can then be used to identify new potential binding sites, given a new set of sequences, and to predict the strength of the TF-DNA binding. Motif discovery can be considered a classification or a regression problem, depending on the type of data used to train M . The datasets derived by experimental assays like ChIP-seq or HT-SELEX provide hundreds or thousands of sequences containing binding sites. In this setting, motif discovery

becomes a classification problem. In fact, the goal is to discriminate between bound and unbound sites in the input sequences, and train the motif model with the identified binding sites. Datasets produced by other experimental technologies like PBM provide the binding strength for large sets of sequences of equal length. Therefore, rather than discriminating between bound and unbound sequences, in this setting the motif model learns the binding affinity values associated to each target site in the input dataset, transforming motif discovery into a regression problem. In both settings the final goal is to derive a computational model M , describing the recovered TFBS and capable of predicting new binding events along with their affinity in sequences not used during model training. The most common models to represent TFBS are *consensus sequences* (Day and McMorris, 1992), *position weight matrices* (PWMs) (Stormo, 2000, 2013), *high-order PWMs* (Siddharthan, 2010; Korhonen *et al.*, 2017), *k-mer-based* (Gorkin *et al.*, 2012), and *deep neural network-based* (He *et al.*, 2021) models. Consensus sequences summarize in a single sequence the TFBS structure, displaying at each position the most frequent nucleotide. However, consensus sequences do not provide the contribution of each nucleotide in each binding site position to the TF-DNA binding. PWMs address this limitation providing the contribution of each nucleotide to TF binding. However, PWMs do not account for potential dependencies among TFBS nucleotides. High-order PWMs extend the PWM model to consider local dependencies between neighboring TFBS positions. K-mer-based models provide the contribution of each k-mer to the TFBS, implicitly integrating dependency among nucleotides and local sequence features in the model. Deep neural network-based motif models integrate in the model complex genomic features governing TF-DNA binding, significantly improving model's performance, but reducing its interpretability.

Enumerative motif discovery algorithms (**Fig.2.2 (B)**) assume that motifs are overrepresented patterns in the input dataset S with respect to a set of background genomic sequences B . Enumerative algorithms may assume that the motif length $|M|$ is known a priori. Let us assume $|M| = k$, the general idea is to collect the approximate occurrences of all potential 4^k k -mers in the sequences of S and assess if the difference between the number of matches found in S and B , or the number of expected matches according to a background model, is statistically significant. Then, an ungapped alignment is built from the statistically significant k -mers to derive a PWM. However, searching the approximate occurrences of all 4^k k -mers quickly becomes impractical, even for small k . Early proposals introduced the usage of heuristics to reduce the search space, like searching exact occurrences in a subset of the input sequences (Li *et al.*, 1999), or restricting mismatching locations to fixed motif positions (Califano, 2000). Since mismatches can occur at any motif position, these solutions partially address the motif discovery problem. The application of indexing data structures like suffix trees (STs) (Weiner, 1973) allowed to explore the entire motif search space, by providing an efficient data structure speeding-up the exhaustive search for motif candidates. Weeder (Pavesi *et al.*, 2001, 2004b) and SMILE (Marsan and Sagot, 2000) extended this idea to consider approximate pattern matches without restrictions on mismatches locations. To assess the statistical significance of motif candidates, SMILE compares the number of motif occurrences found in S with those retrieved in a background dataset. Similarly, Weeder compares the frequency of motif candidates with their expected frequency in promoters region of the same organism from which the input sequences were collected. However, these methods can take several hours to search for long motifs, or analyze large sequence datasets enriched in similar motif occurrences like those produced by PBMs, HT-SELEX, or ChIP methods (Liu *et al.*, 2018). In such datasets is sufficient to allow one or two mismatching positions to recover the TF binding sequences, while Weeder performs an exhaustive search on all k -mers, allowing more than two substitutions, increasing the algorithm running time. Therefore, during the last decades the community proposed several enumerative algorithms specifically designed to analyze the large datasets produced with NGS-based assays. MDscan (Liu *et al.*, 2002) uses word enumeration to find motif candidates in ChIP on Chip datasets. Instead of enumerating all the potential words from the sequences in S , MDscan extracts the non-redundant patterns from the most enriched sequences. To assess the statistical significance of motif candidates, MDscan employs a third-order Markov model as background. Amadeus (Linhart *et al.*, 2008) evaluates all k -mers in S , obtained from ChIP on Chip experiments, and groups the similar patterns in lists. The similar patterns lists are grouped in motifs, which are statistically evaluated using a hypergeometric test. DREME (Bailey, 2011) searches motif candidate sequences using regular expressions. To statistically evaluate the discovered motifs, DREME employs the Fisher's exact test, comparing the number of sequences in S and B , in which the motifs occur. Trawler, HOMER and STREME (Ettwiller *et al.*, 2007; Heinz *et al.*, 2010; Bailey, 2021) use STs similarly SMILE or Weeder. Trawler enumerates all sequence patterns using STs indexing S and B , and measures patterns frequencies to find overrepresented sequences in the input dataset. To match motif sequences, Trawler employs degenerate consensus, allowing mismatching positions. To statistically evaluate the prioritized patterns, Trawlers uses the z -scores, derived from the normal-approximation to the binomial distribution.

Since the function does not correct for the effect of overlapping motif instances, the computations are significantly faster. The similar and significant patterns are clustered to form motifs. HOMER algorithm was specifically designed to discover TFBS motifs in ChIP-seq data. HOMER indexes the foreground and background datasets using STs and searches for k-mers overrepresented in S , through approximate pattern matching on the ST. HOMER employs the hypergeometric or binomial test to statistically evaluate each motif candidate. Using the hypergeometric or the binomial tests reduces the running time, by avoiding the explicit count of k -mers frequencies. STREME builds a ST from S and B , and counts the number of exact matches of seed words of different lengths in both datasets, evaluating the statistical significance of their enrichment using Fisher's exact test or the binomial distribution. Then, STREME counts the number of the approximate matches of the most significant words on the ST. The prioritized words are then grouped to derive the corresponding motifs. Importantly, STREME requires one single tree visit to discover motifs of different lengths, significantly speeding-up the discovery of different TFBS motifs.

Alignment-based motif discovery algorithms compute alignment profiles to describe motif binding preferences (**Fig.2.2 (B)**), avoiding the exhaustive k -mers enumeration. The rationale is to build an alignment from k -mers selected from the input dataset S , and score the resulting profile through appropriate measures, like nucleotide conservation, information content, or profile statistical significance. Motif statistical significance is based on the probability of obtaining the same alignment from random sequences or from a background dataset B . Alignment-based motif discovery algorithms usually assume that the motif length $|M|$ is known *a priori*. For alignment-based algorithms motif discovery can be formalized as a combinatorial problem. Let us assume $|M| = k$, the goal is to find the best alignment profile built combining k -mers from S , according to a scoring criterion. The best alignments are then used to derive the corresponding PWMs. Most alignment-based algorithms assume that each sequence in S contains one or zero binding sites. Therefore, there exist $(\sum_{s \in S} |s| - |M| + 1)^{|S|}$ possible profiles, built by combining k -mers in all possible ways. Since enumerating all possible solutions is computationally impractical even for small dataset, alignment-based algorithms explore the solution space using heuristics, such as greedy (Hertz and Stormo, 1999), expectation-maximization (EM) (Bailey *et al.*, 1994), stochastic (e.g. Gibbs sampling) (Lawrence *et al.*, 1993), or genetic algorithms (Lee *et al.*, 2018). CONSENSUS (Hertz and Stormo, 1999) proposed a greedy approach to incrementally build the motif alignment profiles. The problem is initially solved on two sequences, then is progressively solved by adding the remaining sequences $s \in S$ one by one. The MEME algorithm (Bailey *et al.*, 1994; Bailey and Elkan, 1995; Bailey *et al.*, 2006) proposed a different strategy to explore the solution space by iteratively refining an initial profile, substituting some k -mers in the profile with others more likely to produce better solutions. By employing an EM strategy, MEME evaluates how well each k -mer in $s \in S$ fits the current alignment profile, rather than a background model. The algorithm has two main steps: the E-step and the M-step. During the E-step, MEME computes a likelihood score for each k -mer in S , using the current alignment profile. In the M-step, MEME assigns a weight to each k -mers in the current profile proportional to the scores computed during the E-step and updates the alignment by substituting low scoring k -mers with others better fitting the current profile. However, the EM algorithm can prematurely converge to local maxima and convergence depends on the algorithm starting conditions. Stochastic optimization strategies like Gibbs sampling (Lawrence and Reilly, 1990) address these limitations. Given an initial profile built with k -mers randomly selected from each $s \in S$, at each iteration the algorithm removes from the profile the k -mer coming from a certain s . Then, the algorithm assigns a likelihood score to each k -mer in s using the modified profile. Similarly to MEME, the score describes how well each k -mer fits the profile rather than a background model. Then, a new k -mer from s is chosen to replace the removed subsequence in the profile, with probability proportional to its likelihood score. The procedure is repeated until a fixed number of iterations or no further updates applied to the profile. Therefore, while EM optimization strategies like MEME update the profile selecting the k -mers deterministically according to how well they fit the alignment, Gibbs sampling algorithms optimize the profile selecting the subsequences to remove and insert using a stochastic approach. However, the basic Gibbs sampling algorithm assumes that each sequence contains exactly one binding site. In (Neuwald *et al.*, 1995) the authors extended the algorithm to consider sequences containing one, multiple, or no binding sites. AlignACE (Hughes *et al.*, 2000) and ANN-spec (Workman and Stormo, 1999) furtherly modified the Gibbs sampling algorithm, enabling the simultaneous search for TFBS on both strands. Moreover, ANN-spec coupled the stochastic motif search with a perceptron artificial neural network learning the best alignment from the binding specificities observed in S . BioProspector (Liu *et al.*, 2000) and MotifSampler (Thijs *et al.*, 2001) used third order Markov models as background, improving the predictive performance of Gibbs sampling. GLAM (Frith *et al.*, 2004) modified the Gibbs sampling strategy to estimate the optimal alignment length, and

employed simulated annealing for profile optimization. GLAM algorithm was then furtherly extended to also consider gapped motifs (Frith *et al.*, 2008). Although stochastic and EM are the most popular optimization strategies for alignment-based algorithms, researchers also focused on adopting other optimization methods. GADEM (Li, 2009) paired EM local search with genetic algorithms for profile refinement. However, using alignment profiles the solution search space grows exponentially with the size of S . Even employing heuristics, the analysis of the datasets produced by NGS assays rapidly becomes impractical and requires large amounts of time and computational resources (Zambelli *et al.*, 2013). Therefore, researchers focused on developing new alignment-based motif discovery algorithms tailored to analyze the datasets produced by high-throughput protocols. MEME-ChIP (Machanick and Bailey, 2011) and STEME (Reid and Wernisch, 2011) improved the original MEME algorithm for the analysis of ChIP datasets. MEME-ChIP focuses the analysis on a random subset of sequences in S , reducing the theoretical size of the solution space. STEME indexes the sequences in S using suffix trees and avoids to evaluate the likelihood of all possible k-mers during MEME's E-step, by employing a branch and bound strategy on the ST. ChIPMunk (Kulakovskiy *et al.*, 2010) proposed a greedy profile optimization similar to EM, to discover motifs in large ChIP datasets, while accounting for ChIP peaks shape. ChIPMunk explores the solution space to find the alignment maximizing the profile discrete information content (Kulakovskiy and Makeev, 2009), and uses peaks shape to weight the contribution of each sequence $s \in S$ to the motif. XXmotif (Hartmann *et al.*, 2013) combined enumerative motif discovery with profile refinement, by iteratively selecting sets of k -mers from S maximizing their fitness to the profile, and increasing the motif fitness to S . Similarly, ProSampler (Li *et al.*, 2019) proposed a highly optimized hybrid method to discover motifs in large ChIP-seq datasets, combining motif enumeration with Gibbs sampling to refine preliminary motif profiles.

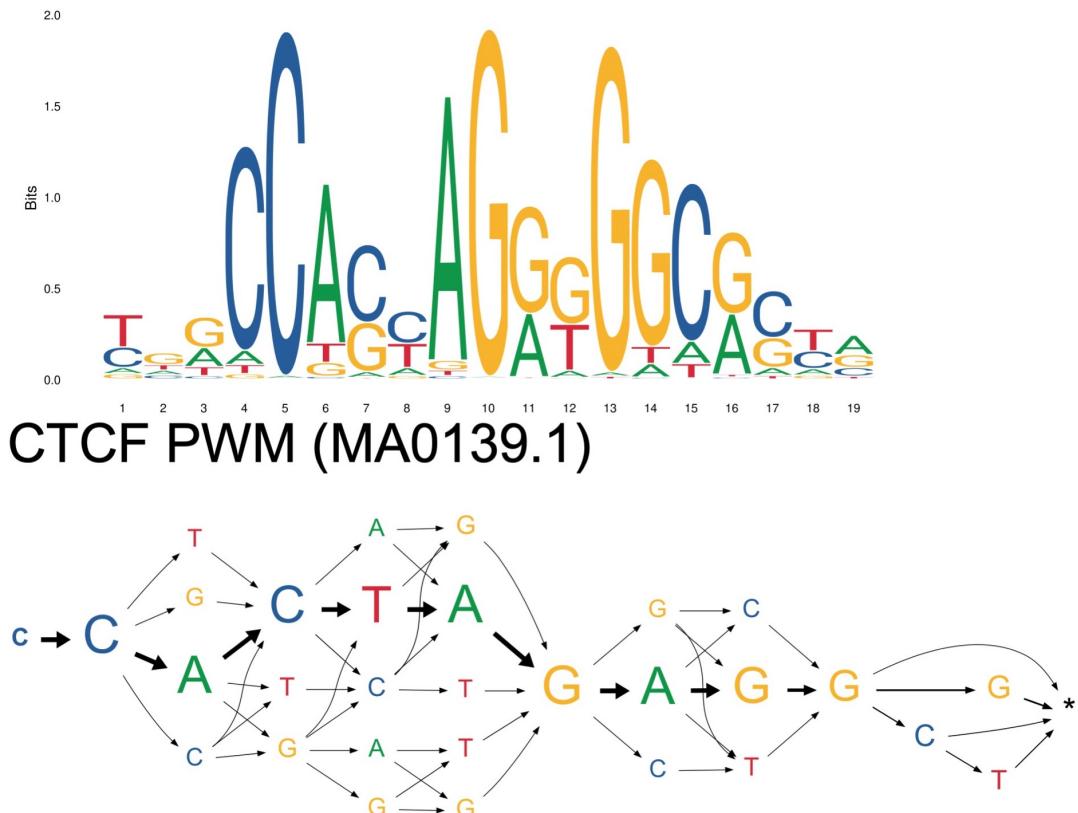
The importance of dependencies between TFBS nucleotides and how including them in motif models would improve the models' performance has been controversially debated (Tomovic and Oakeley, 2007; Morris *et al.*, 2011; Zhao and Stormo, 2011). However, some studies demonstrated the existence of such dependencies in TFBS, between neighboring and non-neighboring nucleotides (Slattery *et al.*, 2014; Rohs *et al.*, 2010). Enumerative and alignment-based algorithms represent motifs without accounting for potential dependencies between the binding site positions, modeling TFBS as PWMs. PWMs can be extended to count the frequency di- or tri-nucleotides, producing high-order PWMs, like dinucleotide weight matrices (DWMs) (Siddharthan, 2010). Dimont (Grau *et al.*, 2013) and diChIPMunk (Kulakovskiy *et al.*, 2013) employed DWMs to discover and represent motifs. However, dependencies may exist also between non-neighboring positions and not between neighboring nucleotides. Therefore, the models could learn dependencies not in the data, often overfitting the training set S . Probabilistic graphical models (**Fig.2.2 (B)**) like Bayesian networks (BNs) or Markov models (MMs) provide flexible and efficient frameworks to capture and encode dependencies between TFBS nucleotides. Barash and coworkers (Barash *et al.*, 2003) proposed to model TFBS motifs as BNs trained through an EM strategy. Importantly, BNs can capture dependencies between neighboring and non-neighboring motif positions. However, the model assumes the same order of dependence throughout the entire binding site. Similarly, in (Ben-Gal *et al.*, 2005) the authors introduced VOBN models representing TFBS as BNs, but accounting for variable orders of dependencies between neighboring and non-neighboring TFBS positions. However, BNs training is often computationally demanding and requires large amounts of data to avoid the model to overfit S . MMs and Hidden Markov models (HMMs) provide efficient frameworks to include dependencies between motif positions, and more scalable and efficient training procedures compared to BNs. TFFMs (Mathelier and Wasserman, 2013) proposed a HMM-based model capturing dinucleotide dependencies between neighboring motif positions. TFFM models also capture the properties of the sequences flanking the binding site and accommodate changes in the motif length, by employing the background and insertion/deletion states, respectively. TFFMs are trained using a set of previously prioritized motif candidate sequences and employing the Baum-Welch algorithm. TFFM models assume the same order of dependency between neighboring positions across the whole TFBS. Slim models (Keilwagen and Grau, 2015) introduced a framework to learn neighboring and non-neighboring dependencies between TFBS nucleotides, using different orders of dependency. Dependency orders are pruned in a data-driven fashion, establishing the TFBS positions on which the motif nucleotides depend on. The models are trained using the motif candidates prioritized using Dimont. Discrover (Maaskola and Rajewsky, 2014) proposed a discriminative motif discovery method designed to analyze ChIP-seq datasets and modeling TFBS as HMMs. Given S and B , Discrover searches for overrepresented motifs in S using regular expressions. Then, an initial HMM is trained using the prioritized motif candidates as seeds. To identify good seeds, Discrover uses different objective functions such as likelihood, or mutual information. The initial HMM is then iteratively refined through a gradient optimization of likelihood. In (Eggeling *et al.*, 2014) the

authors proposed a method to discover CTCF (Bell *et al.*, 1999) motif using variable-order MMs to capture different orders of dependency between neighboring nucleotides. The model is trained using an EM strategy iteratively refining the MM parameters, to optimize its fitness to the input dataset S . BaMMotif (Siebert and Söding, 2016) introduced an efficient method to discover TFBS motifs in large datasets, employing a Bayesian approach to train Markov models. To avoid model overfitting, the motif model is trained using the conditional probabilities of $(q - 1)$ th order as priors for the q th order conditional probabilities. The algorithm iteratively optimizes the model parameters until convergence through an EM strategy. In the E-step, the algorithm estimates the probability that each position of each $s \in S$ is a motif starting position, using the current model. During the M-step, BaMMotif uses the motif candidates identified in the previous step to refine the model. Moreover, the algorithms dynamically adapt the model parameters during training to capture variable orders of dependencies between motif positions. Recently, the authors developed a faster and improved of the algorithm (Ge *et al.*, 2021).

Support Vector Machines (SVMs) (Boser *et al.*, 1992) have been successfully applied to different problems in computational biology (Ben-Hur *et al.*, 2008), including TFBS motif discovery (**Fig.2.2 (B)**). The general idea is to decompose the bound sequences (foreground dataset S) and the unbound sequences (background dataset B) in sequence elements of length k (k -mers). The k -mers frequencies are then used as features to train a sequence similarity kernel (Ben-Hur *et al.*, 2008), to discriminate between bound and unbound sequences. Generally, to each k -mer is assigned a weight proportional to its contribution to the definition of the positive or negative training sets, or to its likelihood of being a motif candidate. Although early k -mer based kernels (Leslie *et al.*, 2001; Eskin *et al.*, 2002; Kuang *et al.*, 2005) were originally developed to tackle protein sequence homology problems, recently several SVM-based algorithms extending the original kernels were proposed to discover TFBS motifs. Kmer-SVM (Lee *et al.*, 2011; Fletez-Brant *et al.*, 2013) proposed a SVM-based method to discover TFBS motifs in ChIP-seq datasets. Kmer-SVM employs the spectrum kernel (Leslie *et al.*, 2001), which counts the exact matches for all contiguous k -mers in S and B , building the k -mers feature space. By using a trie (Bodon and Rónyai, 2003) indexing S and B , the algorithm runs in linear time. Although TFBS motifs contain highly degenerate non-informative positions, kmer-SVM does not explicitly accommodate such sequence variability by counting the frequency of exact k -mers matches.

Motif Graphs

Several studies showed that TFs present population-specific (Kasowski *et al.*, 2010), cell-type-specific (Gertz *et al.*, 2013; Arvey *et al.*, 2012), and even individual-specific (Tognon *et al.*, 2021) binding sites. Algorithms analyzing only the reference genome would provide general models, which could return wrong TFBS predictions when analyzing personal genomic sequences. Motif models integrating data from different populations, cell-types, or individuals would recover more reliable TFBS occurrence predictions (**Fig.3.3 (A)**). Moreover, such models would better predict the consequences of noncoding variants on TF-DNA binding events, and they could encode variable orders of nucleotide dependencies. Importantly, graph data structures are often interpretable and intuitive. With this aims in mind we developed the Motif Graph, a framework integrating the advantages of *probabilistic graphical models-based* and *SVM-based* motif discovery algorithms, without sacrificing model interpretability. A Motif Graph model G is defined by a set of vertices V , a set of edges E , and a set of paths P . Each vertex $v \in V$ on the graph is labeled with a nucleotide, or mathematically $\text{label}(v) \in \{A, C, G, T\}$. Each edge $e \in E$ represents the allowed links between consecutive nucleotides in the TFBS motif. Each path $p \in P$ represents a *haplotype* embedded in G , which correspond to one sequence used to train the Motif Graph model. Currently, the



CTCF motif graph

Figure 3.3. Comparison between CTCF MotifGraph model and its PWM from the JASPAR database. On top the CTCF motif available on JASPAR database (MA0139.1). On the bottom the Motif Graph model trained using 100 k-mers obtained from a ChIP-seq experiment targeting CTCF binding site on HepG2 cell line.

model is limited to encode 1st order dependencies between consecutive nucleotides. However, the model is flexible and accomodate variable length motifs, beside recording the training sequences.

3.1 Motif Graph model construction

Motif Graph motif discovery procedure (**Algorithm 1**) is constituted of two main steps: k -mers prioritization, and graph model training and construction. To prioritize the k -mers Motif Graph currently employs the k -mer based motif discovery procedure implemented in Gkm-SVM (Ghandi *et al.*, 2014). Therefore, given a positive sequence dataset S and a background dataset B , for each k -mer of length k in S , the algorithm counts the number of matches in S and in B , allowing mismatching positions (see **Section 2.1.2** for further details). Then, a similarity kernel is trained using the recovered k -mers frequencies. The trained kernel assigns to each k -mer in S and in B a weight proportional to its contribution in defining the foreground or the background dataset. The algorithm then ranks the k -mers according to their weight scores. The Motif Graph model is iteratively trained employing a greedy approach, which adds the top ranked k -mers to G incrementally, one by one (see **Algorithm 2** for details). Each k -mer is aligned to the current G to maximize the number of nucleotides matching the current model. While aligning the k -mers to the current Motif Graph model, the algorithm shifts the input k -mer on the right and on the left up to a defined offset number of nucleotides. In our experiments with set the offset to 3. Once built the model, we construct a scoring matrix similar to the widely used *PSSM*. However, our scoring matrix account for 1st order dependencies between nucleotides, recalling the well-known DWMs (see **Section 2.1.3** for details). The scoring matrix is used to assign a likelihood score and classify new sequences as potentially bound or not bound by the investigated factor. In other words, the score describes how likely is the scanned sequence to contain a potential binding site. To score a sequence we slide the scoring matrix along the string employing a procedure similar to classical PWM scanning tools like FIMO (Grant *et al.*, 2011).

Algorithm 1: Motif Graph motif discovery.

Input: S, B, k
Output: G

```

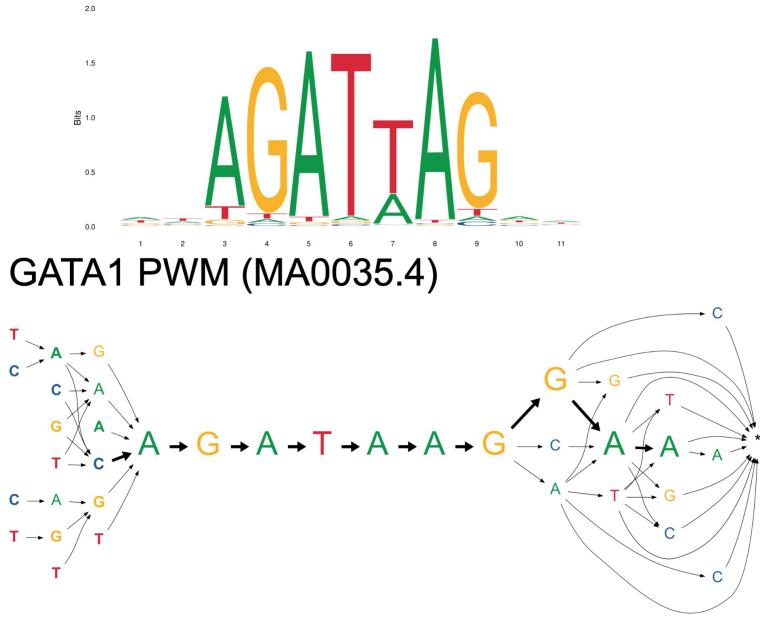
1 frequencies ← countFrequencies( $S, B, k$ )
2 kernel ← trainKernel(frequencies)
3 kmers, weights ← extractWeights(kernel)
4 rankedKmer ← sort(kmers, weights)
5  $G \leftarrow \emptyset$ 
6 for kmer in rankedKmers do
7    $\sqsubset G \leftarrow \text{addKmers}(G, \text{kmer})$ 
8 return  $G$ 
```

Algorithm 2: Motif Graph model training.

Input: G , kmer, i
Output: G

```

1 if  $i = 1$  then
2    $\sqsubset \text{return } G$ 
3 for  $j$  in 1 to 3 do
4    $\sqsubset \text{matchesLeftOffset, alignmnetLeft} \leftarrow \text{countMatchesLeftOffset}(G, \text{kmer}, j)$ 
5 for  $j$  in 1 to 3 do
6    $\sqsubset \text{matchesRightOffset, alignmnetRight} \leftarrow \text{countMatchesRightOffset}(G, \text{kmer}, j)$ 
7  $\text{alignment} \leftarrow \text{getBestAlignment}(\text{matchesLeftOffset, alignmentLeft, matchesRightOffset,}$ 
      $\text{alignmentRight})$ 
8  $G \leftarrow \text{insertKmer}(G, \text{alignment})$ 
9 return  $G$ 
```



GATA1 motif graph

Figure 3.4. Comparison between GATA1 MotifGraph model and its PWM from the JASPAR database. On top the GATA1 motif available on JASPAR database (MA0035.4). On the bottom the Motif Graph model trained using 100 k-mers obtained from a ChIP-seq experiment targeting GATA1 binding site on K562 cell line.

Training k-mers	10	50	100	200	350	500	750
AUC	0.69	0.69	0.72	0.63	0.60	0.55	0.55
F1-score	0.62	0.69	0.71	0.65	0.62	0.57	0.56

Table 3.2. CTCF Motif Graph model AUC and F1-scores values with different number of training k-mers.

3.2 Results

To test our motif discovery algorithm we obtained 10,000 ChIP-seq peak sequences from the ENCODE Project database (Consortium *et al.*, 2012) for CTCF and GATA1 Transcription Factors, obtained on the HepG2 and K562 cell lines, respectively. The original ChIP-seq datasets were sorted according to the peaks enrichment score in decreasing order, in order to test our algorithm on reliable peaks. Interestingly, the trained Motif Graph models were closed to the motifs PWM available on the JASPAR database (Sandelin *et al.*, 2004) for both CTCF and GATA1 (**Fig.3.3** and **Fig.3.4**). For both TFs, the Motif Graph models captured the main motif sequence. The main motifs are also enforced by the edge thickness which is proportional to the number of training k-mers supporting each $p \in P$. Then, we tested the discriminative performance of both Motif Graph model, with different number of training k-mers (**Fig.3.5**), to establish the optimal number of training sequences for the CTCF and GATA1 Motif Graph models. To compare the models discriminative performance, we performed a cross-validation experiment using splitting the S and B dataset in training and testing set (75% and 25%, respectively). We trained the models with 10, 50, 100, 200, 350, 500, and 750 k-mers. For CTCF the best performance in terms of both AUC (0.72) and F1-score (0.71) were obtained training the Motif Graph on 100 k-mers (**Table 3.2**). For GATA1 the model returned the best AUC using 200 k-mers (0.76), while the best F1-score (0.70) was obtained training the model with 100 sequences (**Table 3.3**). Then, we compared the Motif Graph models discriminative performance against the corresponding PWM and DWM models, recovered from JASPAR (Sandelin *et al.*, 2004) and HOCOMOCO (Kulakovskiy *et al.*, 2016) databases, respectively. For CTCF, both the PWM and the DWM models returned better predictive performance than our model (**Fig.3.6 (A)**). On the other hand, on GATA1 data our model showed better performance than PWMs, but still performed worse than DWMs (**Fig.3.6 (B)**).

Training k-mers	10	50	100	200	350	500	750
AUC	0.73	0.75	0.75	0.76	0.74	0.74	0.71
F1-score	0.68	0.68	0.70	0.69	0.70	0.69	0.67

Table 3.3. GATA1 Motif Graph model AUC and F1-scores values with different number of training k-mers.

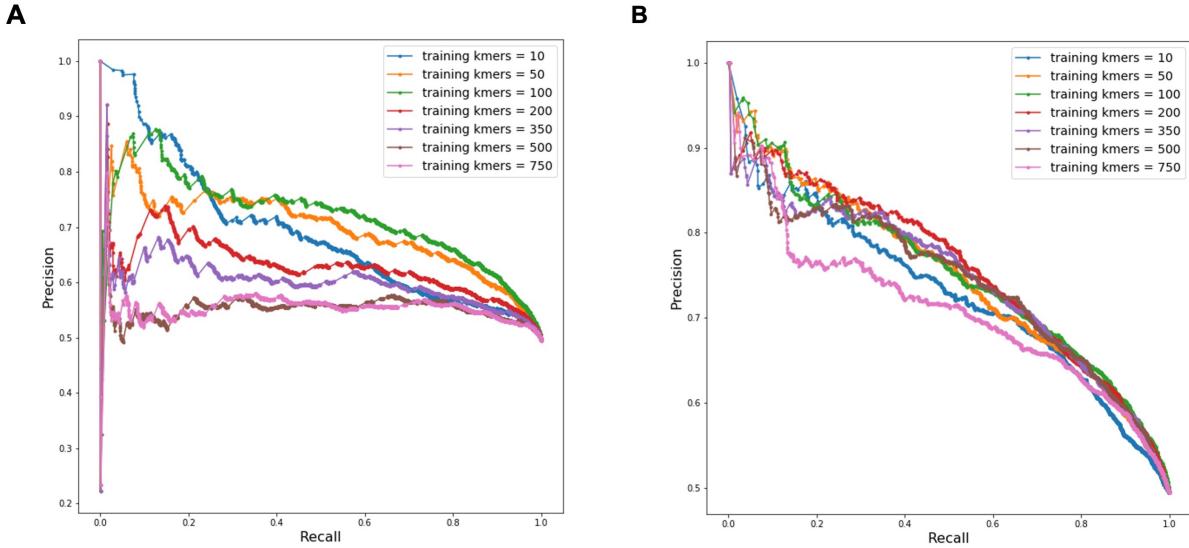


Figure 3.5. Precision-Recall curves obtained varying the number of k -mers used to train the Motif Graph models. To establish the number of training k -mers returning the best discriminative performance we computed the Precision-Recall curves of different Motif Graph models trained using 10, 50, 100, 200, 350, 500, and 750 k -mers on (A) CTCF and (B) GATA1

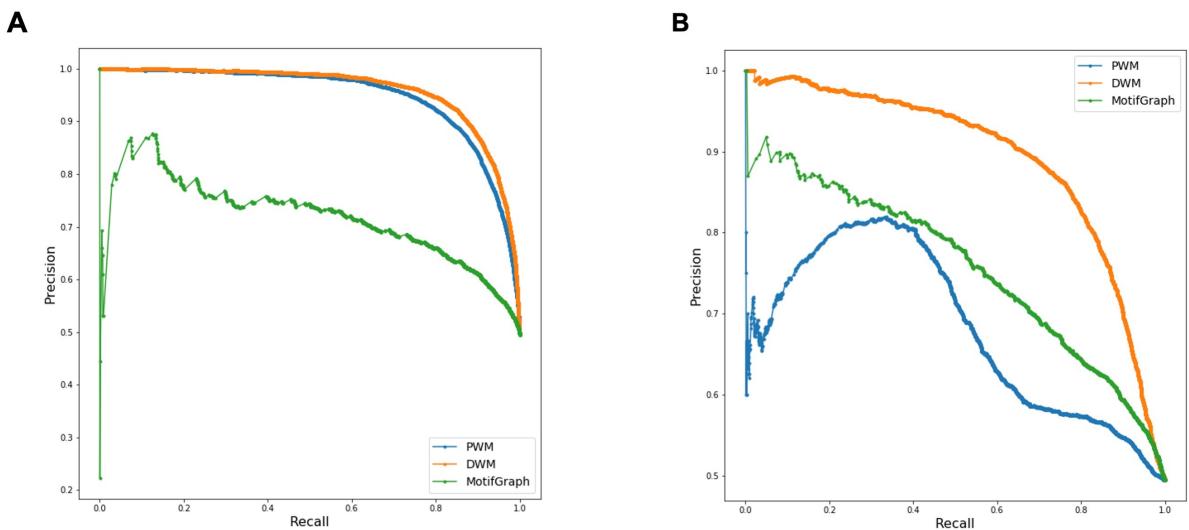


Figure 3.6. Comparing Motif Graph, PWM, and DWM Precision-Recall curves. We compared the discriminative power of the Motif Graph models against that of PWMs and DWMS for both (A) CTCF and (B) GATA1.

Variant effects prediction on Transcription Factor Binding Sites

Several studies showed that genetic variants can significantly impact TF-DNA binding events (De Gobbi *et al.*, 2006; Weinhold *et al.*, 2014; Guo *et al.*, 2018). Genome-wide association studies (GWASs) uncovered thousands of genetic variants (SNPs), associated with complex human traits, located in noncoding regions of the genome. Most of these variants are located within functional regulatory elements, like enhancers (Maurano *et al.*, 2012). Therefore, misregulations in gene expression may be mediated by SNPs modulating TF-DNA interactions. Such variants may perturb TF-DNA binding sequences, potentially changing the downstream gene expression (Deplancke *et al.*, 2016). Importantly, mutations altering TFBS can occur in haplotypes conserved within a population of individuals (Kasowski *et al.*, 2010), resulting in population specific TFBS. Similarly, cell-type genetic variability can create cell-type specific TF target sequences. Therefore, the development of software designed to predict the potential effects of genetic variation on TFBS specificity accounting for haplotype- and cell-type specific mutations becomes fundamental to increase our knowledge about the cell mechanisms governing gene expression. To this aim, we developed two tools predicting mutations effects on TFBS in haplotypes and different cell-types. GRAFIMO (Tognon *et al.*, 2021) is a variant- and haplotype-aware motif scanning tool searching potential occurrences of known TF motifs on genome graphs (Paten *et al.*, 2017). Briefly, genome graphs are graph-based data structures, where nodes correspond to DNA sequences and edges describe allowed links between successive sequences. Paths through the graph, which may be labelled (such as in the case of a reference genome), correspond to haplotypes belonging to different genomes (Sirén *et al.*, 2020) (**Fig.5.7**). Variants like SNPs and indels form bubbles in the graph, where diverging paths through the graph are anchored by a common start and end sequence on the reference [138]. MotifRaptor (Yao *et al.*, 2021) investigates the impact of genetic variants on TF, exploiting cell-type specific information regarding chromatin accessibility and gene expression. While the original MotifRaptor used PWM models to assess genetic variants effects, we extended the framework to integrate k -mer-based motif models (see **Section 2.1.3** for details), which provide more reliable predictions.

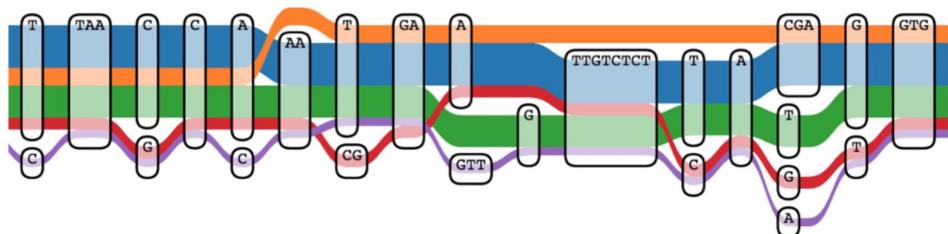


Figure 5.7. Genome Graphs data structure visualization. Each color corresponds to a path in the graph. Each path represents the genomic sequences of one of the individual genomes encoded in the Genome Graph structure.

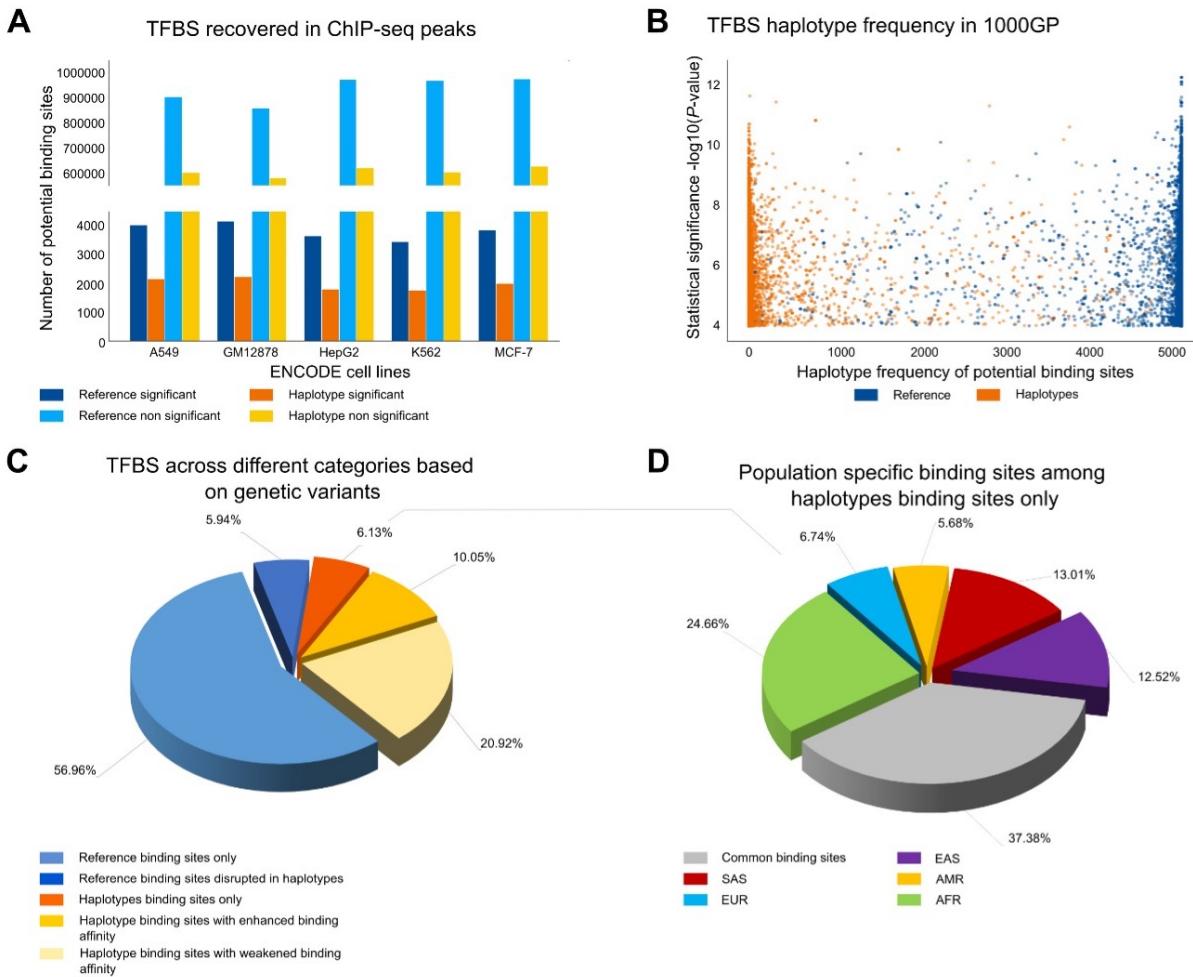


Figure 5.8. Searching CTCF motif on VG with GRAFIMO provides an insight on how genetic variation affects putative binding sites. (A) Potential CTCF occurrences statistically significant ($P\text{-value} \leq 1e-4$) and non-significant found in the reference and in the haplotype sequences found with GRAFIMO on hg38 1000GP VG. (B) Statistical significance of retrieved potential CTCF motif occurrences and frequency of the corresponding haplotypes embedded in the VG. (C) Percentage of statistically significant CTCF potential binding sites found only in the reference genome or alternative haplotypes and with modulated binding scores based on 1000GP genetic variants (D) Percentage of population specific and common (shared by two or more populations) potential CTCF binding sites present on individual haplotypes.

5.1 GRAFIMO

GRAFIMO (GRAph-based Finding of Individual Motif Occurrences) (Tognon *et al.*, 2021) is a command-line tool to scan known TF DNA motifs represented as PWMs in genome variation graphs (VGs) (Garrison *et al.*, 2018), a scalable and efficient implement of genome graph framework. GRAFIMO extends the standard PWM scanning procedure by considering variations and alternative haplotypes encoded in a VG. Using GRAFIMO on a VG based on individuals from the 1000 Genomes project (Siva, 2008) we recover several potential binding sites that are enhanced, weakened or missed when scanning only the reference genome, and which could constitute individual-specific binding events. During the last decade, several methods have been developed to search TFBS on linear reference genomes, such as FIMO (Grant *et al.*, 2011) and MOODS (Korhonen *et al.*, 2009) or to account for SNPs and short indels such as is-rSNP, TRAP and atSNP (Macintyre *et al.*, 2010; Thomas-Chollier *et al.*, 2011; Zuo *et al.*, 2015), however these tools do not account for individual haplotypes nor provide summary on the frequency of these events in a population. We observed that several CTCF motif occurrences are found only in non-reference haplotypes, therefore a consistent number of potential motif occurrences are potentially lost when scanning only the reference sequence (Fig.5.8 (A)). Moreover, we found several potential CTCF occurrences with high statistical significance occurring in rare haplotypes, which could modulate gene expression in those subjects (Fig.5.8 (B)). We also investigated how often regulatory mutations disrupt, create or modulate TFBS binding affinity. Interestingly, we observed that many TFBS can be found only on individual haplotypes. Moreover, 6% of TFBS are disrupted (do not survive the ss threshold) by the presence of genetic variants, while 30% of potential CTCF TFBS are still significant in

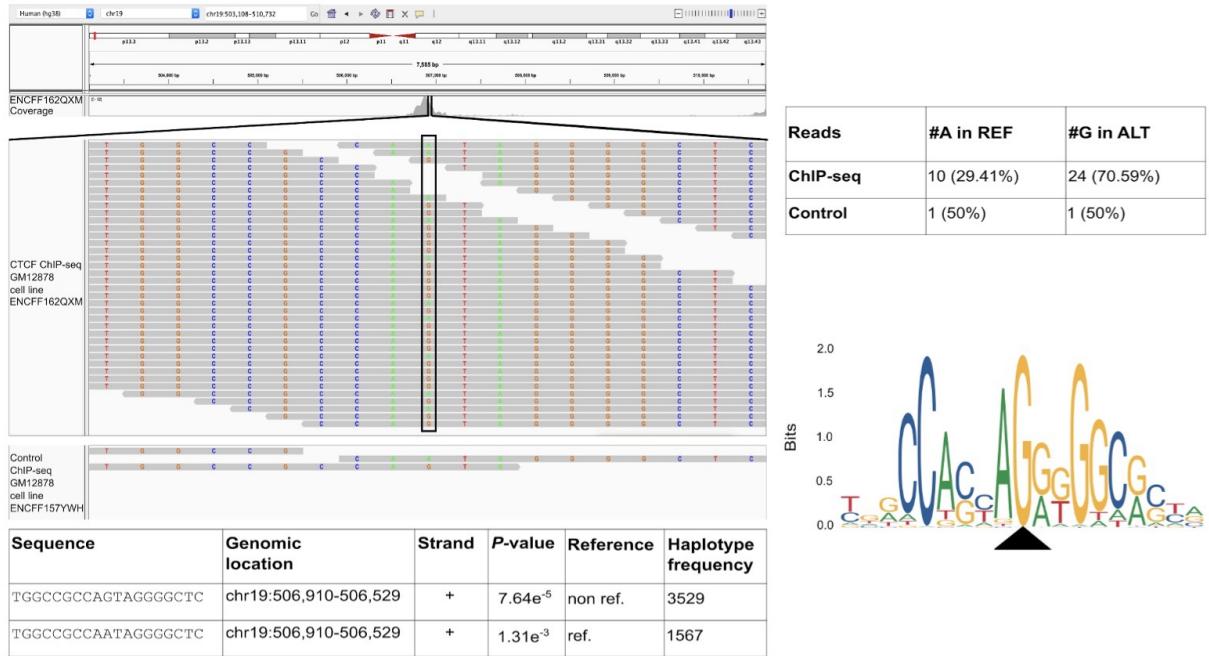


Figure 5.9. Considering genomic variation, GRAFIMO captures more potential binding events. GRAFIMO reports a potential CTCF binding site at chr19:506,910-506,929 found only in haplotype sequences, searching the motif in ChIP-seq peaks called on cell line GM12878 (experiment code ENCSR000DZN). The reads used to call for ChIP-seq peaks (ENCCFF162QXM) show an allelic imbalance at position 10 of the motif sequence towards the alternative allele G, instead of the reference allele A. The imbalance is captured by GRAFIMO which reports the sequence presenting G at position 10 (found in the haplotypes), while the potential TFBS on the reference carrying an A is not reported as statistically significant (P -value $< 1e-4$). CTCF motif logo shows that the G is the dominant nucleotide in position 10.

non-reference haplotypes, but with enhanced or weakened ba score (Fig.5.8 (C)). We also observed that several putative non-reference TFBS are population-specific (Fig.5.8 (D)). Among the unique CTCF motif occurrences found only on non-reference haplotypes in CTCF ChIP-seq peaks we uncovered one TFBS (chr19:506,910-506,929) that clearly illustrates the danger of only using reference genomes for motif scanning. Within this region we recovered a heterozygous SNP that overlaps (position 10 of the CTCF matrix) and significantly modulates the binding affinity of this TFBS. In fact, by inspecting the ChIP-seq reads (experiment ENCSR000DZN, GM12878 cell line), we observed a clear allelic imbalance towards the alternative allele G (70.59% of reads) with respect to the reference allele A (29.41% of reads). This allelic imbalance is not observed in the reads used as control (experiment code ENCSR000EYX) (Fig.5.9). Taken together these results highlight the importance of considering non-reference genome data when searching for potential TFBS or to characterize their potential activity in a population of individuals.

5.2 MotifRaptor2

Several studies showed that genetic variants can enhance or disrupt TF-DNA binding affinities. Moreover, most of the SNPs (~ 90%) associated to common diseases and complex phenotypical traits through GWAS analyses have been located within REs, such as enhancers or promoters. MotifRaptor exploits cell-type specific data, like chromatin accessibility and gene expression, to predict the impact of SNPs prioritized via GWAS on TFBS sequence and the corresponding TF-DNA interactions. MotifRaptor workflow is currently constituted of three steps (Fig.5.10). (i) Characterize important cell-types through the enrichment of the phenotype associated SNPs in open chromatin regions. (ii) Search TFBS whose binding potential is significantly modulated by the previously prioritized variants, in the investigated cell-types. (iii) Identify and visualize individual TF-SNP modulation events. The original MotifRaptor employed TFBS PWM models to predict SNP impact on the binding sites of the investigated factor. However, PWMs have several limitations, like the assumption of independence between neighboring and non-neighboring nucleotides within TF target sites, and often they require large amount of training data to return reliable models (Zhao and Stormo, 2011) (see Section 2.1.3 for details). K-mer-based motif models address some of PWMs' limitations. (i) K-mers are features that are either present or not in

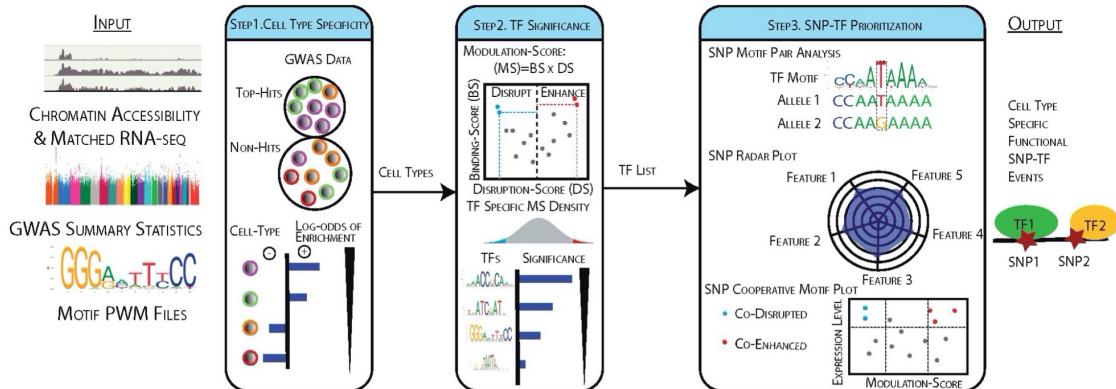


Figure 5.10. MotifRaptor analysis workflow. Three steps are performed: (1) characterize relevant cell types based on the enrichment of phenotype associated SNPs in chromatin accessible sites, (2) find TFs with binding sites that are significantly modulated by genetic variants in these cell types and (3) identify and visualize individual TF-SNP regulation events

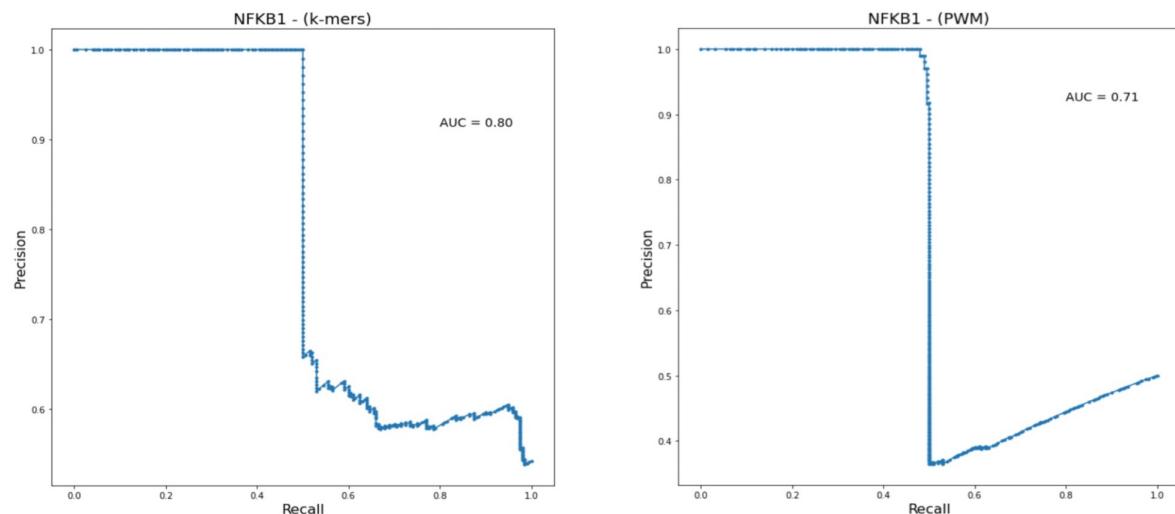


Figure 5.11. Precision-Recall curves for NFKB1 models (*k*-mers and PWM) and AUC.

the training dataset, therefore, the resulting models do not require large amount of training data. (ii) *K*-mers capture *k*th order dependencies between neighboring nucleotides. Therefore, we extended the MotifRaptor analysis to consider *k*-mer-based motif models to assess the impact of genetic variants on TFBS. With this aim we integrated Gkm-SVM analysis pipeline in MotifRaptor framework, to compute sequence scores reflecting SNPs impact on binding sites. We tested MotifRaptor2 by predicting how NFKB1, RUNX3 and STAT1 binding landscapes are affected by genetic variation, considering SNPs linked to rheumatoid arthritis trait. The *k*-mer based models for the investigated TFs were obtained using Gkm-SVM on ChIP-seq peaks sequences from the ENCODE Project database. Interestingly, we observed that *k*-mer-based motif models show a higher discriminative power than PWM when identifying bound and unbound sequences (ChIP-seq peak and random sequences, respectively) (Fig.5.11). Therefore, MotifRaptor2 is a promising tool to produce reliable predictions regarding genetic variants impact on transcription factors binding landscapes.

CRISPR off-targets

CRISPR gene editing (Cong *et al.*, 2013) enabled the genetic engineering of the genomes of living organisms. CRISPR provides a simple and programmable platform coupling the binding to genomic target sequences with diverse effector proteins restricted by protospacer adjacent motif (PAM) sequences. By delivering the Cas9 nuclease complexed with a synthetic guide RNA (gRNA) into a cell, CRISPR provides a simple and programmable platform to modify the genomic sequence at a desired location, potentially allowing the removal or addition of genes *in vivo*. Importantly, CRISPR offers unprecedented opportunities to develop novel therapies by introducing targeted genetic or epigenetic modifications to the genomic regions of interest. CRISPR-Cas9 offers high fidelity and simple construction and its specificity depends on two factors: (i) the target sequence and (ii) the PAM sequence. The target sequence is 20 bp long as part of each CRISPR locus in the gRNA array (Ran *et al.*, 2013). Typically, crRNA has multiple unique targets. Cas9 selects the genomic location by pairing the gRNA with its complementary sequence on the host DNA. Since the gRNA sequence is not part of the Cas9 complex, it can be designed independently to target specific genomic locations (Bialk *et al.*, 2015). To exploit the exonucleasic function Cas9 recognize its PAM sequences. PAMs are very short nonspecific sequences, occurring in several locations along the genome (Ran *et al.*, 2013). Once assembled the required sequences, Cas9 finds the targets on the genome, guided by the gRNA. The Cas9 nuclease opens both genomic strands of the target sequence to introduce novel modifications in it. Cas9 works in two main methods: (i) knock-in, and (ii) knock-out mutations (**Fig.6.12**). In knock-in, homology directed repair (HDR) employs DNA sequences similar to the targets to repair the breaks in the genome caused by Cas9 exonucleasic actions, using exogenous DNA as repairing template. Importantly, this method relies on periodic and isolated damaged spots in the target sites to start the DNA repair operations. In knock-out, mutations in the DNA inserted by Cas9 result in the repair of breaks through nonhomologous end joining (NHEJ). DNA repair via NHEJ often results in random insertions and deletions in the target sequence, which may disrupt, enhance, or alter the function of the target site. Since CRISPR-Cas9 enables a targeted random gene disruption, designing gRNA finely guiding Cas9 to the desired target sequence (*on-targets*) is fundamental to avoid unexpected and dangerous outcomes on undesired targeted sequences (*off-targets*). Most importantly, genetic variants may alter protospacer and PAM sequences and may influence both on-target and off-target potential. Therefore, it is fundamental to consider genetic variability when designing gRNA, to avoid potential undesired and dangerous outcomes on the host genome, in particular in clinical settings.

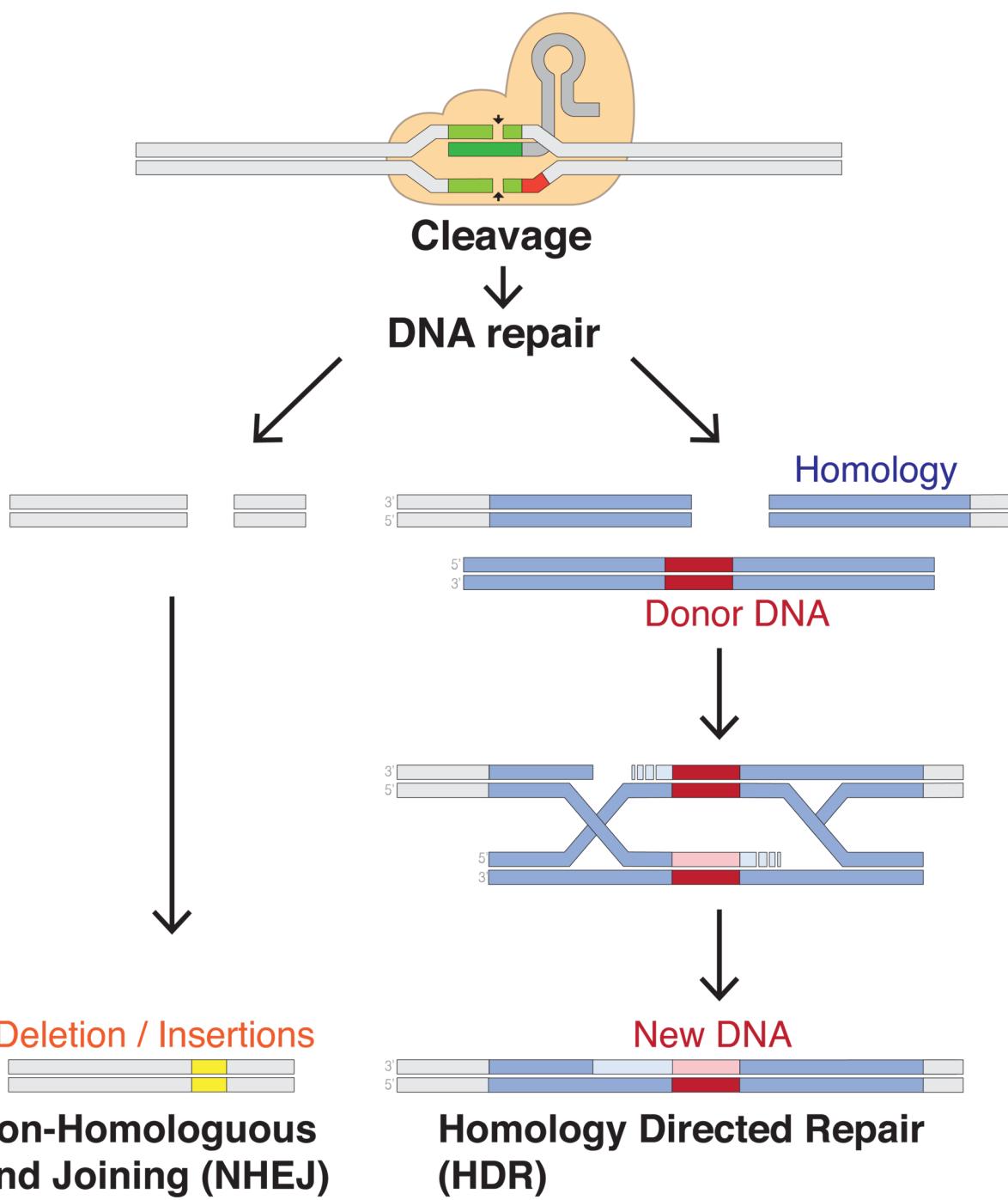


Figure 6.12. CRISPR gene editing via HDR and NHEJ.

CRISPRme

CRISPR-based systems may create unintended off-target modifications posing potential genotoxicity for therapeutic use. Several experimental assays and computational methods are available to uncover or forecast these off-targets (Clement *et al.*, 2020). Off-target sites are partially predictable based on homology to the spacer and PAM sequence. Beyond the number of mismatches or bulges, a variety of sequence features, like position of mismatch or bulge with respect to PAM or specific base changes, contribute to off-target potential (Clement *et al.*, 2020; Bao *et al.*, 2021; Hsu *et al.*, 2013; Doench *et al.*, 2016). Computational models can complement experimental approaches to off-target nomination in several respects: to triage gRNAs prior to experiments by predicting the number and cleavage potential of off-target sites and to prioritize target sites for experimental scrutiny. Gene editing strategies designed to specifically recognize patient mutations may increase the likelihood of editing mutant alleles, whereas variants that reduce homology to the anticipated target may decrease the efficiency of the desired genetic modification. Although a variety of in vitro and cell-based experimental methods can be used to empirically nominate off-target sites, these methods either use homology to the reference genome as a criterion to define the search space and/or use a limited set of human donor genomes to evaluate off-target potential (Bao *et al.*, 2021; Chaudhari *et al.*, 2020). Therefore, computational methods may be especially useful to predict the impact of off-target sequences not found in reference genomes. Prior studies considering gRNAs targeting therapeutically relevant genes using population-based variant databases like the 1000 Genomes Project (1000G) (Siva, 2008) and the Exome Aggregation Consortium (Karczewski *et al.*, 2017) have highlighted how genetic variants can significantly alter the off-target landscape by creating novel and personal off-target sites not present in a single reference genome (Lessard *et al.*, 2017; Scott and Zhang, 2017). Although these prior studies provide code to reproduce analyses, implementation choices make these tools not suitable to analyze large variant datasets and to consider higher numbers of mismatches. In addition, these methods ignore bulges between RNA:DNA hybrids, cannot efficiently model alternative haplotypes and indels, and require extensive computational skills to utilize. Several user-friendly websites have been developed to aid the design of gRNAs and to assess their potential off-targets (Concordet and Haeussler, 2018; Listgarten *et al.*, 2018; Labun *et al.*, 2019; Park *et al.*, 2015). Even though variant-aware prediction is an important problem for genome editing interventions, these scalable graphical user interface (GUI) based tools do not account for genetic variants. In addition, these tools artificially limit the number of mismatches for the search and/or do not support DNA/RNA bulges. Therefore, designing gRNAs for therapeutic intervention using current widely available tools could miss important off-target sites that may lead to unwanted genotoxicity. A complete and exhaustive off-target search with an arbitrary number of mismatches, bulges, and genetic variants that is haplotype-aware is a computationally challenging problem that requires specialized and efficient data structures. We have recently developed a command line tool that partially solves these challenges called CRISPRitz (Cancellieri *et al.*, 2020). This tool uses optimized data structures to efficiently account for single variants, mismatches and bulges but with significant limitations (Cancellieri *et al.*, 2020). We substantially extended CRISPRitz by developing CRISPRme (Cancellieri *et al.*, 2022), a tool to aid gRNA design with added support for haplotype-aware off-target enumeration, short indel variants and a flexible number of mismatches and bulges. CRISPRme is a unified, user-friendly web-based application that provides several reports to prioritize putative off-targets based on their risk in a population or individuals. CRISPRme is flexible to accept user-defined genomic annotations, which could include empirically identified off-target sites or cell type specific chromatin features. It can integrate population genetic variants from sets of phased individual variants (like those from 1000G), unphased individual variants (like those from the Human Genome Diversity Project, HGDP (Bergström *et al.*, 2020)) and population-level variants (like those from the Genome Aggregation Database, gnomAD (Karczewski *et al.*, 2020)). Furthermore, it can accept personal genomes from individual subjects to identify and prioritize private off-targets due to variants specific to a single individual. Here we demonstrate the utility of CRISPRme by analyzing the off-target potential of

a gRNA currently being tested in clinical trials for SCD and β -thalassemia (Frangoul *et al.*, 2021; Canver *et al.*, 2015; Wu *et al.*, 2019). We identify possible off-targets introduced by genetic variants included within and extending beyond 1000G. We predict that the most likely off-target site, overlooked by prior analyses, is introduced by a variant common in African-ancestry individuals (rs114518452, minor allele frequency (MAF)= 4.5%) and provide experimental evidence of its off-target potential in gene edited human CD34+ hematopoietic stem and progenitor cells. Furthermore, we demonstrate that alternative allele-specific off-target potential is widespread across various gRNAs and editors.

7.1 CRISPRme: a variant-aware computational tool to nominate candidate off-target sites

CRISPRme is a web-based tool to predict off-target potential of CRISPR gene editing that accounts for genetic variation. CRISPRme can also be deployed locally as a web app or used as a command line utility, both of which respect genomic privacy offline. CRISPRme takes as input a Cas protein, gRNA spacer sequence(s) and PAM, genome build, sets of variants (VCF files for populations or individuals), user-defined thresholds of mismatches and bulges, and optional user-defined genomic annotations to produce comprehensive and personalized reports (**Fig.7.13 (A)**). We designed CRISPRme to be flexible with support for new gene editors with variable and extremely relaxed PAM requirements (Walton *et al.*, 2020). Thanks to a PAM encoding based on Aho-Corasick automata and an index based on a ternary search tree, CRISPRme can perform genome-wide exhaustive searches efficiently even with an NNN PAM, extensive mismatches (tested with up to 7) and RNA:DNA bulges (tested with up to 2). Notably, a comprehensive search performed with up to 6 mismatches, 2 DNA/RNA bulges and a fully non-restrictive PAM (NNN) takes only 24 hours on a small computational cluster node (Intel Xeon CPU E5-2609 v4 clocked at 2.2 GHz and 128 GB RAM). All the 1000G variants, including both SNVs and indels, can be included in the search together with all the available metadata for each individual (sex, super-population and age), and the search operation takes into account observed haplotypes. Importantly, off-target sites that represent alternative alignments to a given genomic region are merged to avoid inflating the number of reported sites. Although several tools exist to enumerate off-targets, to our knowledge only two command line tools (Lessard *et al.*, 2017; Fennell *et al.*, 2021) incorporate genetic variants in the search. However, they have several limitations in terms of scalability to large searches, number of mismatches, bulges, haplotypes, and variant file formats supported and do not provide an easy-to-use graphical user interface. CRISPRme generates several reports. (i) It summarizes for each gRNA all the potential off-targets found in the reference or variant genomes based on their mismatches and bulges (**Fig.7.13 (B)**) and generates a file with detailed information on each of these candidate off-targets. (ii) It compares gRNAs to customizable annotations. By default, it classifies possible off-target sites based on GENCODE22 (genomic features) and ENCODE23 (candidate cis-regulatory elements, cCREs) annotations. It can also incorporate user-defined annotations in BED format, such as empiric off-target scores or cell type specific chromatin features (**Fig.7.14**). (iii) Using 1000G and/or HGDP16 variants, CRISPRme reports the cumulative distribution of homologous sites based on the reference genome or super-population. These global reports could be used to compare a set of gRNAs based on how genetic variation impacts their predicted on- and off-target cleavage potential using cutting frequency determination (CFD) or CRISPR Target Assessment (CRISTA) (Abadi *et al.*, 2017) scores (**Fig.7.15**). CRISPRme includes multiple scoring metrics and can be easily extended with new ones, including scores tailored for different editors. Finally, CRISPRme can generate personal genome focused reports called personal risk cards. These reports highlight private off-target sites due to unique genetic variants.

7.2 Results

We tested CRISPRme with a gRNA (#1617) targeting a GATA1 binding motif at the +58 erythroid enhancer of BCL11A18,19. A recent clinical report described two patients, one with SCD and one with β -thalassemia, each treated with autologous gene modified hematopoietic stem and progenitor cells (HSPCs) edited with Cas9 and this gRNA, who showed sustained increases in fetal hemoglobin, transfusion-independence and absence of vaso-occlusive episodes (in the SCD patient) following therapy. This study as well as prior pre-clinical studies with the same gRNA (#1617) did not reveal evidence of off-target editing in treated cells when considering off-target sites nominated by bioinformatic analysis of the human reference genome and empiric analysis of in vitro genomic cleavage potential (Frangoul

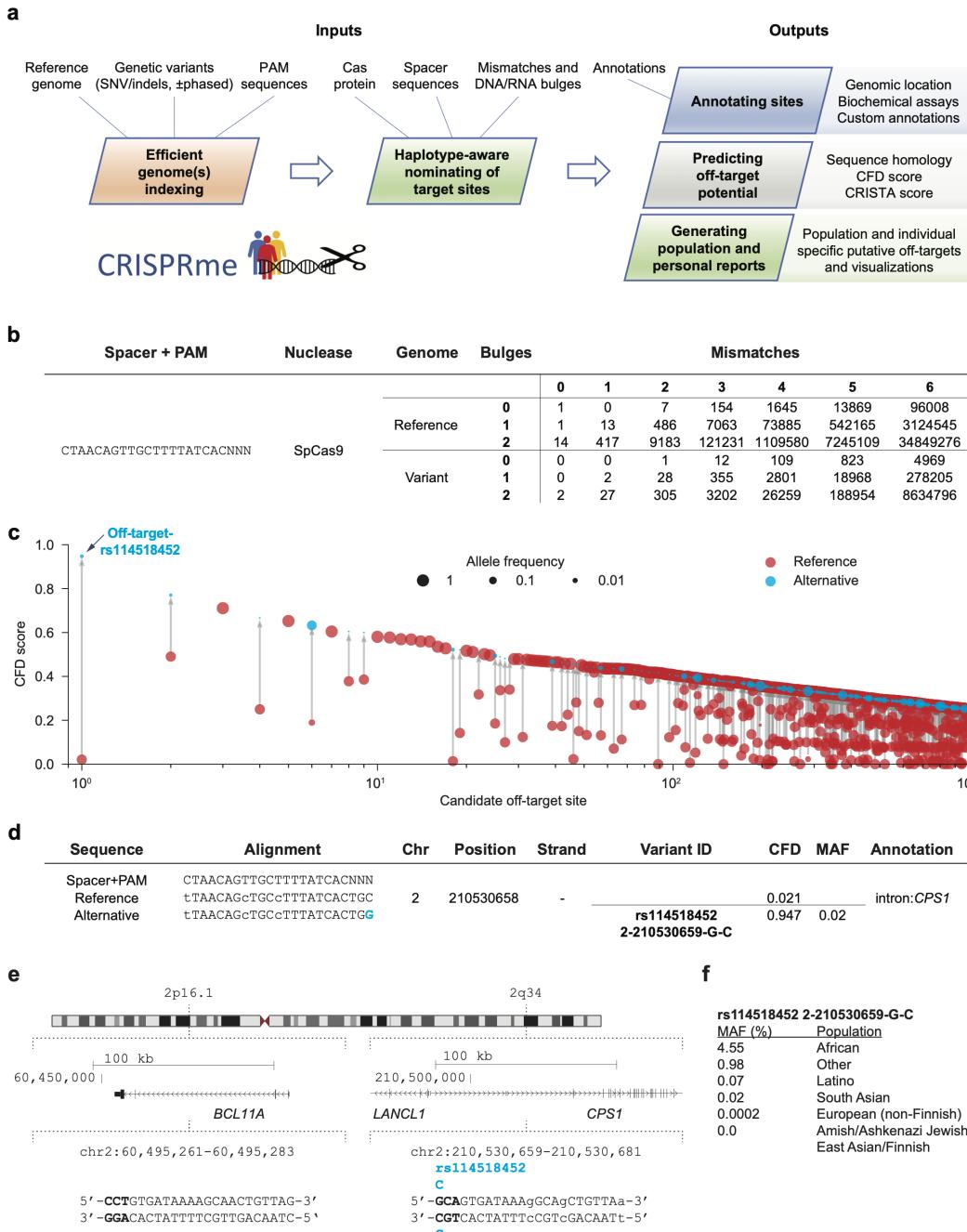


Figure 7.13. CRISPRme provides web-based analysis of CRISPR-Cas gene editing off-target potential reflecting population genetic diversity. (A) CRISPRme software takes as input a reference genome, genetic variants, PAM sequence, Cas protein type, spacer sequence, homology threshold and genomic annotations and provides comprehensive, target-focused and individual-focused analyses of off-target potential. It is available as an online webtool and can be deployed locally or used offline as command-line software. (B) Analysis of the BCL11A-1617 spacer targeting the +58 erythroid enhancer with SpCas9, NNN PAM, 1000G variants, up to 6 mismatches and up to 2 bulges. (C) Top 1000 predicted off-target sites ranked by CFD score, indicating the CFD score of the reference and alternative allele if applicable, with allele frequency indicated by circle size. (D) The off-target site with the highest CFD score is created by the minor allele of rs114518452. Coordinates are for hg38 and 0-start for the potential off-target and 1-start for the variant-ID. MAF is based on 1000G. (E) The top predicted off-target site from CRISPRme is an allele-specific off-target with 3 mismatches to the BCL11A-1617 spacer sequence, where the rs114518452-C minor allele produces a de novo NGG PAM sequence. PAM sequence shown in bold and mismatches to BCL11A-1617 shown as lowercase. Coordinates are for hg38 and 1-start. (F) rs114518452 allele frequencies based on gnomAD v3.1. Coordinates are for hg38 and 1-start. Spacer shown as DNA sequence for ease of visual alignment.

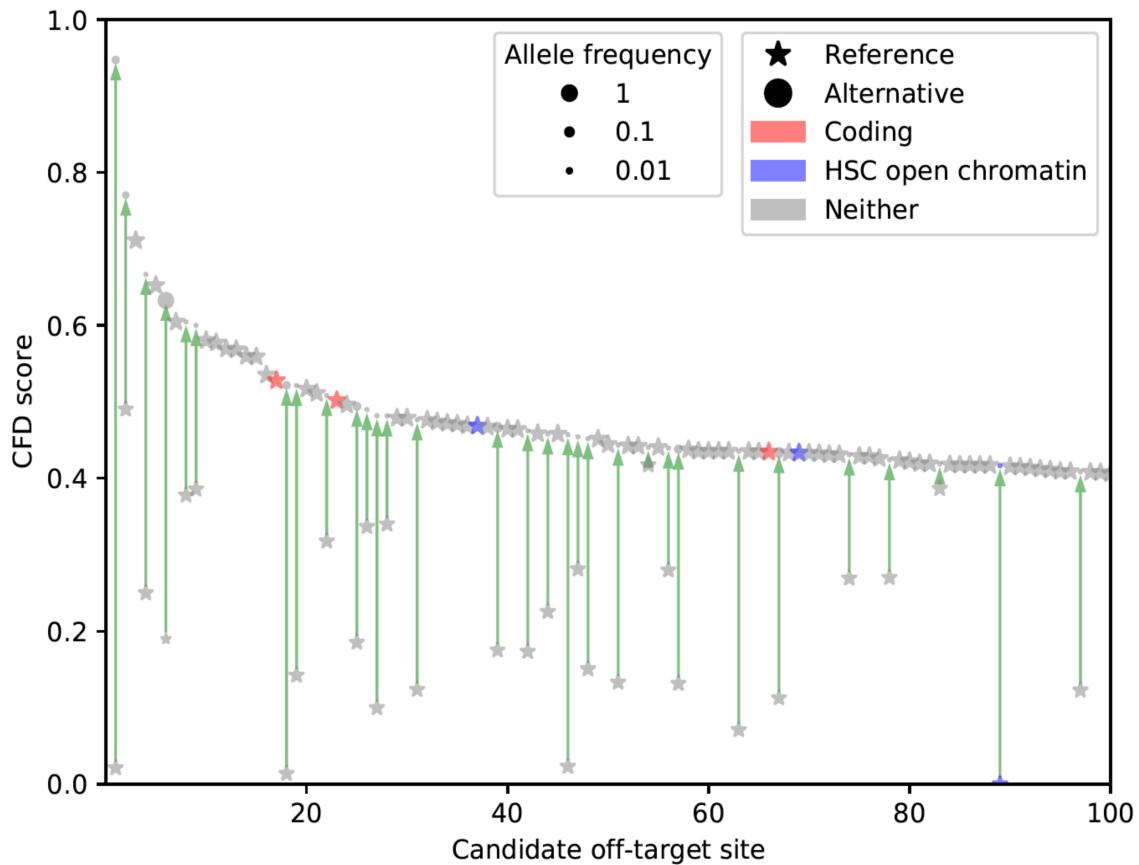


Figure 7.14. Top 100 predicted off-target sites for BCL11A-1617 spacer by CFD score. CRISPRme search as in Fig.7.13. Candidate off-target sites within coding regions based on GENCODE annotations and ATAC-seq peaks in HSCs based on user-provided annotations are highlighted.

et al., 2021; Wu *et al.*, 2019; Demirci *et al.*, 2019). CRISPRme analysis found that the predicted off-target site with both the greatest CFD score and the greatest increase in CFD score from the reference to alternative allele was at an intronic sequence of CPS1 (Fig.7.13 (C) and (D)), a genomic target subject to common genetic variation (modified by a SNP with MAF $\geq 1\%$). CFD scores range from 0 to 1, where the on-target site has a score of 1. The alternative allele rs114518452-C generates a TGG PAM sequence (that is, the optimal PAM for SpCas9) for a potential off-target site with 3 mismatches and a CFD score (CFDalt 0.95) approaching that of the on-target site (Fig.7.13 (E)). In contrast, the reference allele rs114518452-G disrupts the PAM to TGC, which markedly reduces predicted cleavage potential (CFDref 0.02). rs114518452-C has an overall MAF of 1.33% in gnomAD v3.1, with MAF of 4.55% in African/African-American, 0.9% in Other, 0.07% in Latino/Admixed American, 0.02% in European (non-Finnish) and 0.00% in East Asian populations (Fig.7.13 (F)). To consider the off-target potential that could be introduced by personal genetic variation that would not be predicted by 1000G variants, we analyzed HGDP variants identified from whole genome sequences of 929 individuals from 54 diverse human populations. We observed 249 candidate off-targets for gRNA #1617 with CFD ≥ 0.2 for which the CFD score in HGDP exceeded that found for either the reference genome or 1000G variants by at least 0.1 (Fig.7.16 (A) and Fig.7.17). These additional variant off-targets not found from 1000G were observed in each super-population, with the greatest frequency in the African super-population (Fig.7.16 (B)). 229 (92.0%) of these variant off-targets were unique to a super-population and 172 (69.1%) of these were private to just one individual (Fig.7.16 (C)). Furthermore, single individual focused searches, for example an analysis of HGDP01211, an individual of the Oroqen population within the East Asian super-population, showed that most variant off-targets (with higher CFD score than reference) were due to variants also found in 1000G ($n=32369$, 90.4%), a subset were due to variants shared with other individuals from HGDP but absent from 1000G ($n=3177$, 8.9%), and a small fraction were private to the individual ($n=234$, 0.7%) (Fig.7.16 (D)). Among these private off-targets was one generated by a variant (rs1191022522, 3-99137613-A-G, gnomAD v3.1 MAF 0.0053%) where the alternative allele produces a canonical NGG PAM that increases the CFD score from 0.14 to 0.54 (Fig.7.16 (D) and (E)). To experimentally test the top predicted off-target from CRISPRme, we identified a CD34+

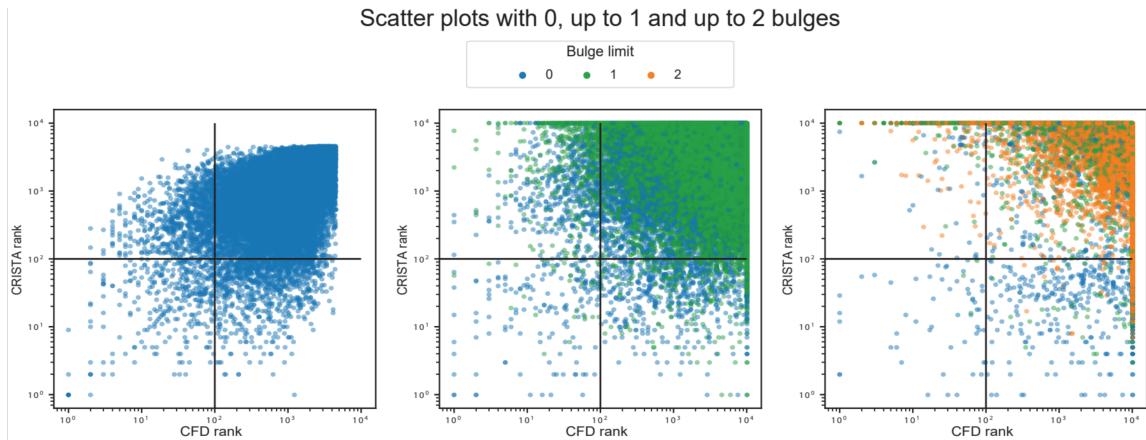


Figure 7.15. Plots with rank ordered correlation between CFD and CRISTA reported targets. Scatter plots show from left to right, the correlation of ranked targets, extracted by selecting top 10000 targets ordered by CFD and CRISTA score, respectively. The left plot shows the rank correlation of targets with 0 bulges (Pearson's correlation: 0.57, $p < 1e^{-10}$, Spearman's correlation: 0.55, $p < 1e^{-10}$), the center plot shows rank correlation of targets with 1 bulge (Pearson's correlation: -0.16, $p < 1e^{-10}$, Spearman's correlation: -0.33, $p < 1e^{-10}$) and the right plot shows the rank correlation of targets with 2 bulges (Pearson's correlation: -0.55, $p < 1e^{-10}$, Spearman's correlation: -0.80, $p < 1e^{-10}$). The correlation values and P -values(two-sided) were calculated using standard functions from the Python scipy library. The colors represent the lowest count of bulges for each target, since the two scoring methods may prioritize different alignments and thus different number of mismatches and bulges of the same genomic target.

HSPC donor of African ancestry heterozygous for rs114518452-C, the variant predicted to introduce the greatest increase in off-target cleavage potential (**Fig.7.13 (C-F)**). We performed RNP electroporation using a gene editing protocol that preserves engrafting HSC function. Amplicon sequencing analysis showed $92.0 \pm 0.5\%$ indels at the on-target site and $4.8 \pm 0.5\%$ indels at the off-target site. For reads spanning the variant position, indels were strictly found at the alternative PAM-creation allele without indels observed at the reference allele (**Fig.7.18 (A-C)**), suggesting $9.6 \pm 1.0\%$ off-target editing of the alternative allele. In an additional 6 HSPC donors homozygous for the reference allele rs114518452-G/G, $0.00 \pm 0.00\%$ indels were observed at the off-target site, suggesting strict restriction of off-target editing to the alternative allele (**Fig.7.18 (D)**). The on-target BCL11A intronic enhancer site is on chr2p while the off-target-rs114518452 site is on chr2q within an intron of a non-canonical transcript of CPS1. Inversion PCR demonstrated inversion junctions consistent with the presence of ~ 150 Mb pericentric inversions between BCL11A and the off-target site only in edited HSPCs carrying the alternative allele (Fig. 4a,b). Deep sequencing of the inversion junction showed that inversions were restricted to the alternative allele in the heterozygous cells (**Fig.7.19 (C)** and (**D**)). Droplet digital PCR revealed these inversions to be present at $0.16 \pm 0.04\%$ allele frequency (**Fig.7.19 (E)**). Various high-fidelity Cas9 variants may improve the specificity of gene editing, although at the possible cost of reduced efficiency (Schmid-Burgk *et al.*, 2020). Gene editing following the same electroporation protocol using a HiFi variant 3xNLS-SpCas9 (R691A) (Vakulskas *et al.*, 2018) in heterozygous cells revealed $82.3 \pm 1.6\%$ on-target indels with only $0.1 \pm 0.1\%$ indels at the rs114518452-C off-target site, i.e. a ~ 48 -fold reduction compared to SpCas9 (**Fig.7.18 (C)**). Inversions were not detected following HiFi-3xNLS-SpCas9 editing (**Fig.7.19 (B)** and (**E**)).

To examine the pervasiveness of alternative allele off-target potential, we evaluated an additional 13 gRNAs in clinical development or otherwise widely used for SpCas9-based nuclease or base editing (Xu *et al.*, 2017, 2019a; Stadtmauer *et al.*, 2020; Gillmore *et al.*, 2021; DeWitt *et al.*, 2016; Xu *et al.*, 2019b; Métais *et al.*, 2019; Tsai *et al.*, 2015; Zeng *et al.*, 2020; Musunuru *et al.*, 2021) and 6 gRNAs for non-SpCas9-based editing such as for SaCas9 and Cas12a (Xu *et al.*, 2019b; Chu *et al.*, 2021; Newby *et al.*, 2021; Maeder *et al.*, 2019; De Dreuzy *et al.*, 2019). CRISPRme analysis including the 1000G and HGDP genetic variant datasets showed 18% (95% confidence interval 13 – 23%) of the total nominated off-targets were due to alternative allele-specific off-targets. Most alternative allele-specific off-targets were associated with rare variants (MAF < 1%), although candidate off-targets associated with common variants were identified for each gRNA (**Fig.7.20 (A)**). None of these alternative allele-specific off-target sites were described in the original manuscripts reporting the editing strategies and off-target analyses. CRISPRme produces visualizations to specifically highlight alternative allele-specific candidate off-target sites overlapping candidate cis-regulatory elements and protein coding sequences (including putative tumor suppressor genes (Zhao *et al.*, 2016)) and/or which involve PAM creation events (**Fig.7.20 (B-C)**). For example, within the top 20 candidate off-targets nominated by CRISPRme for a SpCas9 gRNA tar-

getting EMX135, two sites involve genetic variants with high MAF (52% and 26%) and are associated with substantial increases in CFD score from REF to ALT (+0.69 and +0.44). The first is an intronic PAM creation variant, while the second introduces two PAM-proximal matches to the gRNA (**Fig.7.20 (D)**). Notably, both of these candidate off-targets involve indel variants, underscoring the utility of CRISPRme to account for variants beyond SNPs. In addition to visualizing candidate off-target sites by predictive score rank (such as CFD or CRISTA) for SpCas9 derived editors, CRISPRme can also visualize candidate off-targets by number of mismatches and bulges, which may be especially useful for Cas proteins with distinct PAMs for which predictive scores are not readily available. For example, SaCas9 is a clinically relevant nuclease whose small size favors packaging to AAV. For a SaCas9-associated gRNA targeting CEP29040 currently being evaluated in clinical trials to treat a form of congenital blindness (NCT03872479), CRISPRme nominated two candidate off-targets associated with common SNPs (MAF 7% and 5%) that reduced mismatches from 5 (REF) to 4 (ALT) which are predicted to produce cleavages within coding sequences (**Fig.7.20 (D)**). CRISPRme can nominate variant off-targets for base editors and evaluate their base editing susceptibility within a user-defined editing window. For a gRNA targeting PCSK937 that has been used with SpCas9-nickase adenine base editor in vivo in preclinical studies to reduce LDL cholesterol levels, 4 of the top 5 candidate off-target sites involve alternative alleles, including one with CFDref 0.2 and CFDalt 0.75 found in an ENCODE candidate enhancer element. CRISPRme nominated a candidate off-target associated with a rare variant (MAF 0.0007%) that increased the CFD score from 0.06 (REF) to 0.40 (ALT) which would be predicted to produce missense mutations in EPHB3, a putative tumor suppressor gene (**Fig.7.20 (D)**). The underlying computational challenge that CRISPRme addresses extends beyond CRISPR-based applications to other technologies based on nucleic acid sequence recognition. For example, CRISPRme can nominate off-targets for RNA-targeting strategies, whether RNA-guided gene editors or even oligonucleotide sequences used as RNA interference (RNAi) or antisense oligo (ASO) therapies (**Fig.7.21**). We performed a variant-aware search (without PAM restriction) for the FDA-approved antisense oligonucleotide Nusinersen (Finkel *et al.*, 2017; Mercuri *et al.*, 2018), which targets SMN2 pre-mRNA to treat spinal muscular atrophy. Using CRISPRme, we identified a potential off-target site within a coding region wherein a common SNP (MAF 2%) reduces the number of mismatches from 3 (REF) to 2 (ALT). Similarly, analysis of the FDA-approved RNAi therapy Inclisiran (Raal *et al.*, 2020), which targets PCSK9 mRNA to treat hypercholesterolemia, revealed that its antisense strand has a candidate off-target in the 3' UTR of the ribosomal gene RPP14 for which a common insertion variant (MAF 36%) reduces the number of mismatches and bulges from 7 (REF) to 4 (ALT).

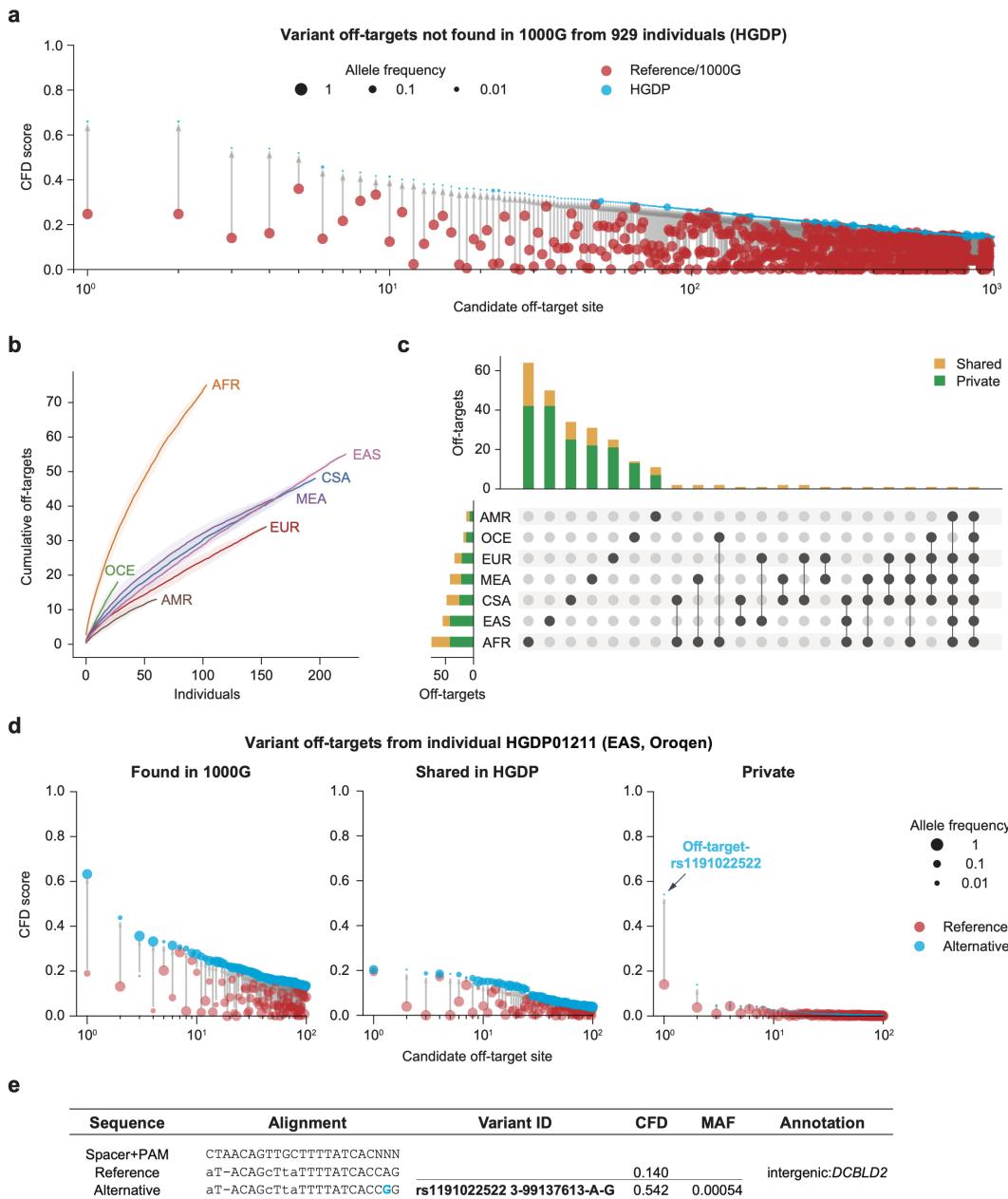


Figure 7.16. CRISPRme provides analysis of off-target potential of CRISPR-Cas gene editing reflecting population and private genetic diversity. (A) CRISPRme analysis was conducted with variants from HGDP comprising whole genome sequencing of 929 individuals from 54 diverse human populations. HGDP variant off-targets with greater CFD scores than the reference genome or 1000G were plotted and sorted by CFD score, with HGDP variant off-targets shown in blue and reference or 1000G variant off-targets shown in red. (B) HGDP variant off-targets with CFD ≥ 0.2 and increase in CFD of ≥ 0.1 . Individual samples from each of the seven super-populations were shuffled 100 times to calculate the mean and 95% confidence interval. (C) Intersection analysis of HGDP variant off-targets with CFD ≥ 0.2 and increase in CFD of ≥ 0.1 . Shared variants were found in 2 or more HGDP samples while private variants were limited to a single sample. (D) CRISPRme analysis of a single individual (HGDP01211) showing the top 100 variant off-targets from each of the following three categories: shared with 1000G variant off-targets (left panel), higher CFD score compared to reference genome and 1000G but shared with other HGDP individuals (center panel), and higher CFD score compared to reference genome and 1000G with variant not found in other HGDP individuals (right panel). For the center and right panels, reference refers to CFD score from reference genome or 1000G variants. (E) The top predicted private off-target site from HGDP01211 is an allele-specific off-target where the rs1191022522-G minor allele produces a canonical NGG PAM sequence in place of a noncanonical NAG PAM sequence. Spacer shown as DNA sequence for ease of visual alignment.

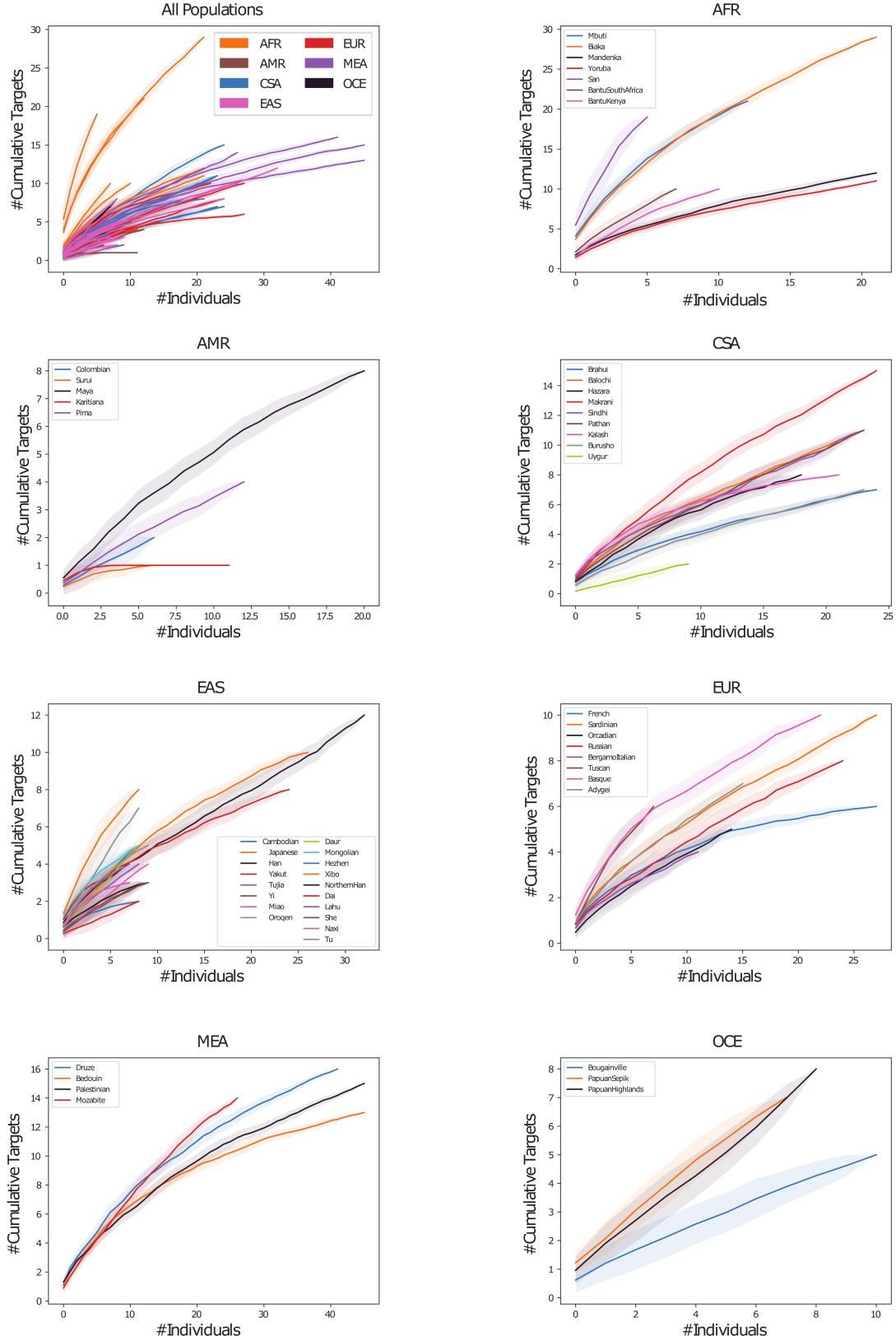


Figure 7.17. HGDP super-population distribution plots. HGDP variant off-targets with CFD ≥ 0.2 and increase in CFD of ≥ 0.1 . Individual samples from each of the seven super-populations were shuffled 100 times to calculate the mean and 95% confidence interval. First panel shows distribution within all 54 discrete populations, colored by super-population. Additional seven panels show distribution of discrete populations within each listed super-population.

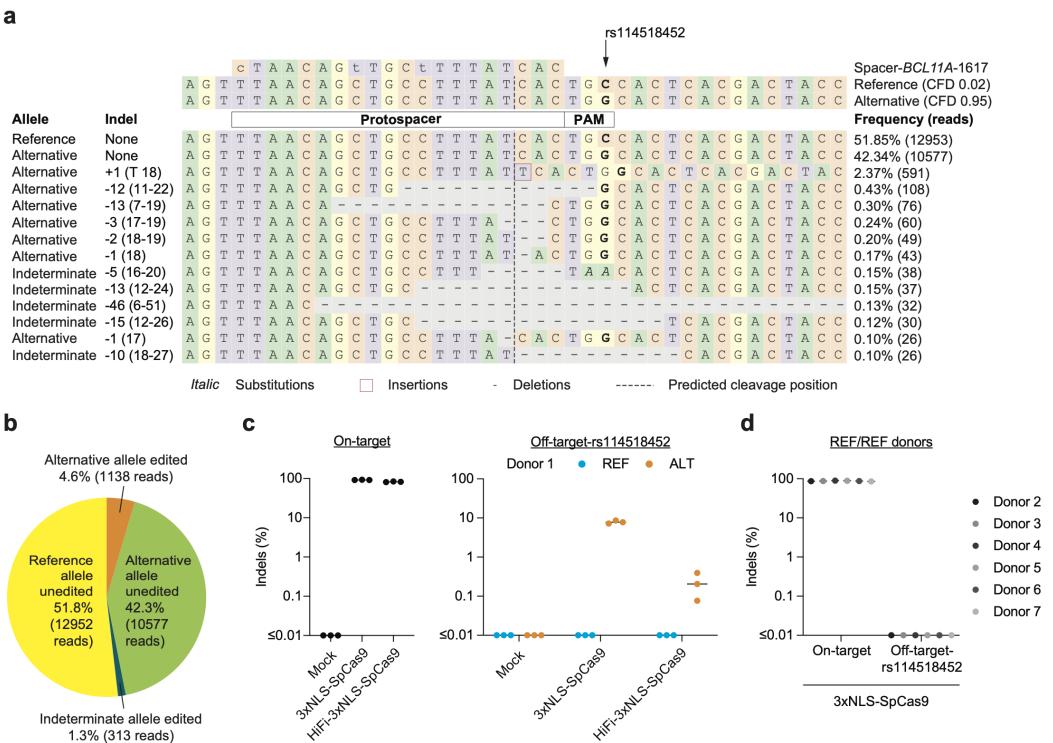


Figure 7.18. Allele-specific off-target editing by a BCL11A enhancer targeting gRNA in clinical trials associated with a common variant in African-ancestry populations. (A) Human CD34+ HSPCs from a donor heterozygous for rs114518452-G/C (Donor 1, REF/ALT) were subject to 3xNLS-SpCas9:sg1617 RNP electroporation followed by amplicon sequencing of the off-target site around chr2:210,530,659-210,530,681 (off-target-rs114518452 in 1-start hg38 coordinates). CFD scores for the reference and alternative alleles are indicated and representative aligned reads are shown. Spacer shown as DNA sequence for ease of visual alignment, with mismatches indicated by lowercase and the rs114518452 position shown in bold. (B) Reads classified based on allele (indeterminate if the rs114518452 position is deleted) and presence or absence of indels (edits). (C) Human CD34+ HSPCs from a donor heterozygous for rs114518452-G/C (Donor 1) were subject to 3xNLS-SpCas9:sg1617 RNP electroporation, HiFi-3xNLS-SpCas9:sg1617 RNP electroporation, or no electroporation (mock) followed by amplicon sequencing of the on-target and off-target-rs114518452 sites. Each dot represents an independent biological replicate ($n = 3$). Indel frequency was quantified for reads aligning to either the reference or alternative allele. (D) Human CD34+ HSPCs from 6 donors homozygous for rs114518452-G/G (Donors 2-7, REF/REF) were subject to 3xNLS-SpCas9:sg1617 RNP electroporation with 1 biological replicate per donor followed by amplicon sequencing of the on-target and off-target-rs114518452 sites.

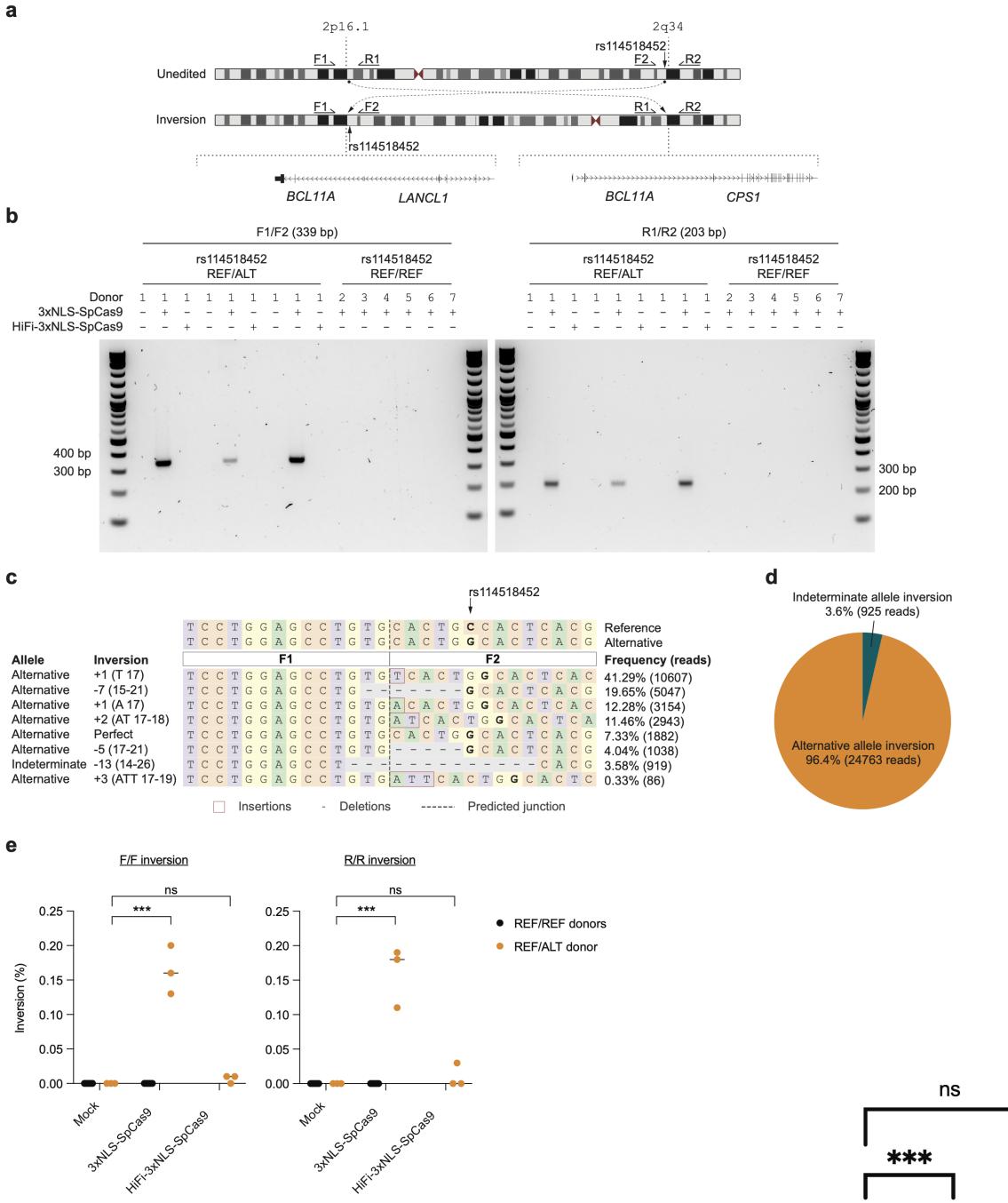


Figure 7.19. Allele-specific pericentric inversion following BCL11A enhancer editing due to off-target cleavage. (A) Concurrent cleavage of the on-target and off-target-rs114518452 sites could lead to pericentric inversion of chr2 as depicted. PCR primers F1, R1, F2, and R2 were designed to detect potential inversions. (B) Human CD34+ HSPCs from a donor heterozygous for rs114518452-G/C (Donor 1) were subject to 3xNLS-SpCas9:sg1617 RNP electroporation, HiFi-3xNLS-SpCas9:sg1617 RNP electroporation, or no electroporation with 3 biological replicates. Human CD34+ HSPCs from 6 donors homozygous for rs114518452-G/G (Donors 2-7, REF/REF) were subject to 3xNLS-SpCas9:sg1617 RNP electroporation with 1 biological replicate per donor. Gel electrophoresis for inversion PCR was performed with F1/F2 and R1/R2 primer pairs on left and right respectively with expected sizes of precise inversion PCR products indicated. (C) Reads from amplicon sequencing of the F1/F2 product (expected to include the rs114518452 position) from 3xNLS-SpCas9:sg1617 RNP treatment were aligned to reference and alternative inversion templates. The rs114518452 position is shown in bold. (D) Reads classified based on allele (indeterminate if the rs114518452 position deleted). (E) Inversion frequency by ddPCR from same samples as in (B). F/F indicates forward and R/R reverse inversion junctions as depicted in (A).

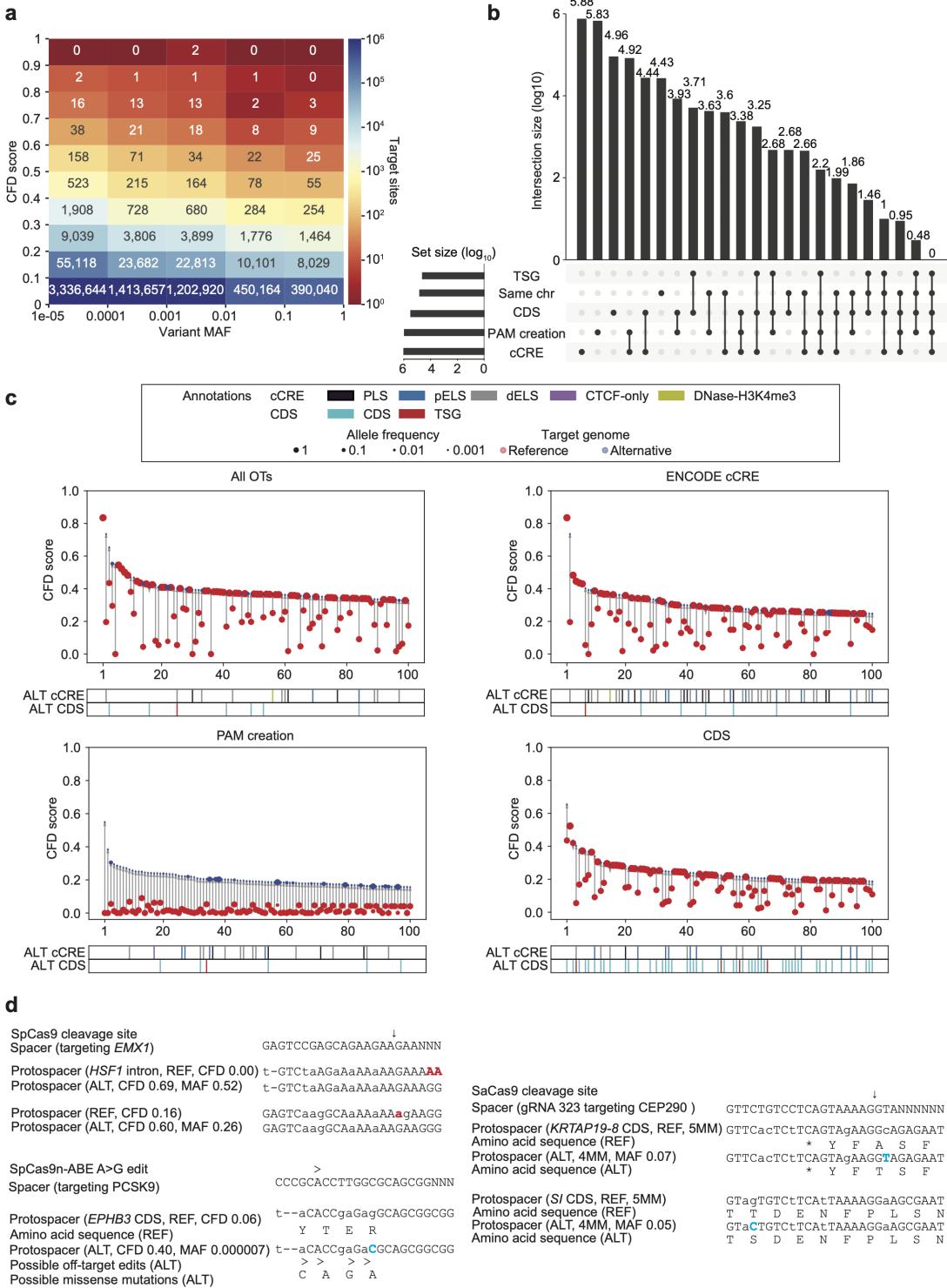


Figure 7.20. CRISPRme illustrates prevalent off-target potential due to genetic variation. (A) Heatmap showing the distribution of alternative allele nominated off-targets for SpCas9 guides by CFD score and MAF. (B) UpSet plot showing overlapping annotation categories for candidate off-targets (TSG, tumor suppressor gene; candidate off-targets on the same chromosome as the on-target; CDS regions; cCRE from ENCODE and PAM creation events). (C) Top 100 predicted off-target sites ranked by CFD score for the gRNA targeting PCSK9 with no filter, found in cCREs, corresponding to PAM creation events, and in CDS regions) (D) Top left: Candidate off-target sites with increased predicted cleavage potential introduced by common (MAF 52% and 26%) indel variants for a SpCas9 gRNA targeting EMX1. Right: Candidate off-target cleavage sites within coding sequences with increased homology to a lead gRNA for SaCas9 targeting of CEP290 to treat congenital blindness in current clinical trials due to common SNPs. Bottom: Potential missense mutations in the EPHB3 tumor suppressor resulting from candidate off-target A-to-G base editing by a preclinical lead gRNA targeting PCSK9 to reduce LDL cholesterol levels. Deletions shown in red, SNPs shown in blue.

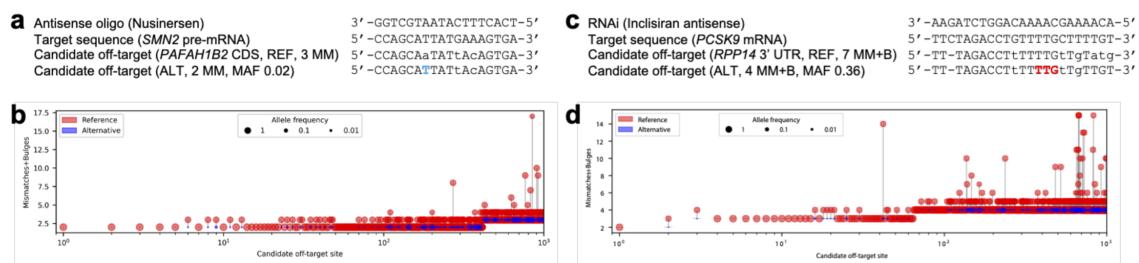


Figure 7.21. Candidate transcript off-targets introduced by common genetic variants for non-CRISPR sequence-based RNA-targeting therapeutic strategies. (A) A common SNP (in blue) introduces a candidate CDS off-target site with 2 mismatches for the FDA-approved antisense oligo Nusinersen. (B) Top 1000 candidate transcript off-targets ranked by mismatches and bulges for Nusinersen from a search performed with the 1000G and HGDP genetic variant datasets. (C) A common insertion variant (in red) introduces a candidate 3'UTR off-target site with 4 mismatches + bulges for the FDA-approved RNAi therapy Inclisiran. (D) Top 1000 candidate transcript off-targets ranked by mismatches and bulges for Inclisiran from a search performed with the 1000G and HGDP genetic variant datasets.

Future directions

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