Regulatory variants: from detection to predicting impact

Elena Rojano, Pedro Seoane, Juan A. G. Ranea and James R. Perkins

Corresponding author: James R. Perkins. Research Laboratory, IBIMA-Regional University Hospital of Malaga, UMA, Malaga 29009, Spain. E-mail: jimrperkins@gmail.com

Abstract

Variants within non-coding genomic regions can greatly affect disease. In recent years, increasing focus has been given to these variants, and how they can alter regulatory elements, such as enhancers, transcription factor binding sites and DNA methylation regions. Such variants can be considered regulatory variants. Concurrently, much effort has been put into establishing international consortia to undertake large projects aimed at discovering regulatory elements in different tissues, cell lines and organisms, and probing the effects of genetic variants on regulation by measuring gene expression. Here, we describe methods and techniques for discovering disease-associated non-coding variants using sequencing technologies. We then explain the computational procedures that can be used for annotating these variants using the information from the aforementioned projects, and prediction of their putative effects, including potential pathogenicity, based on rule-based and machine learning approaches. We provide the details of techniques to validate these predictions, by mapping chromatin–chromatin and chromatin–protein interactions, and introduce Clustered Regularly Interspaced Short Palindromic Repeats-Associated Protein 9 (CRISPR-Cas9) technology, which has already been used in this field and is likely to have a big impact on its future evolution.

We also give examples of regulatory variants associated with multiple complex diseases. This review is aimed at bioinformaticians interested in the characterization of regulatory variants, molecular biologists and geneticists interested in understanding more about the nature and potential role of such variants from a functional point of views, and clinicians who may wish to learn about variants in non-coding genomic regions associated with a given disease and find out what to do next to uncover how they impact on the underlying mechanisms.

Key words: variant analysis; regulatory variants; non-coding DNA; complex diseases; GWAS

Background

Thanks to improvements in sequencing technologies, it is now cheaper and easier than ever to sequence patient genomes with the aim of identifying variants associated with disease. Until recently, researchers were largely interested in variants that overlapped protein-coding regions of the genome. However, results from genome-wide association studies (GWAS) have found more than 88% of disease-associated variants to be in non-coding regions.

Elena Rojano is a Predoctoral Researcher at the Department of Molecular Biology and Biochemistry at the University of Malaga. Her research interests include the development of bioinformatics software for genotype–phenotype associations and regulatory variant analysis.

Pedro Seoane is a Postdoctoral Researcher at the Supercomputing and Bioinnovation Center (SCBI) in the University of Malaga. His research interests include the developing of automatized workflows and bioinformatics software for the analysis of next-generation sequencing data.

Juan Antonio García-Ranea is a Postdoctoral Researcher at the Department of Molecular Biology and Biochemistry in the University of Malaga. His research includes the application of systems biology to study the genetic mechanisms underlying complex and rare diseases.

James Richard Perkins is a Postdoctoral Researcher at the IBIMA Research Laboratory in the Regional University Hospital of Malaga. His research interests include the analysis of genetic and transcriptomic data related to immunological disorders, with a focus on the functional impact of variants in non-coding regions.

Submitted: 13 March 2018; Received (in revised form): 18 April 2018

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non-coding regions [1]. Accordingly, in the past 5 years, a great deal of research has been put into analysis of these regions, although their importance was already increasing thanks to initiatives such as the ENCODE project, whose pilot phase was released over 10 years ago [2, 3]. There are myriad ways by which variants in non-coding regions can affect disease, most of which involve the disruption of genomic elements that regulate gene expression, which we will refer to as regulatory elements [4]. These include cis- and trans-regulatory elements that bind transcription factors (TFs) and other proteins, such as enhancers or promoters [5], and transcribed non-coding regions with regulatory roles, such as micro RNAs (miRNAs) [6] and long non-coding RNAs (lncRNAs) [7]. We will refer to variants in non-coding regions that can affect regulation as regulatory variants.

The procedure for identifying regulatory variants associated with disease is complex and involves both laboratory procedures and computational resources. Some steps are the same as for coding variant identification, such as genome-sequencing, computational analysis of the resultant data and identification of associated variants, for example by comparing allelic frequencies between individuals. For regulatory variant identification, the next steps involve annotation of overlapping regions and predicting their functional effects using a variety of software tools. There are currently around 20 tools available to perform these steps, each with their own advantages, disadvantages and peculiarities. Once regulatory variants have been identified using these tools, an experimental validation procedure is necessary to confirm their predicted impact. An overview of the different steps involved in the identification of regulatory variants associated with disease, from patient to confirmation, is shown in Figure 1.

In this review, we describe this entire process in detail, including how to detect variants using sequencing techniques, determining which of these are disease-associated, searching for overlap of these variants with regulatory elements and predicting their potential impact on the disease based on features of the overlapping region. We will also describe subsequent experimental approaches for validation. In addition, we will present previous studies that have identified and characterized regulatory variants associated with disease by following similar procedures to those described here, including for complex diseases such as coronary artery disease (CAD) [8], Crohn’s disease (CD) [9], schizophrenia [10] and cancer [11]. However, before we discuss these procedures, we must first introduce the main non-coding regulatory elements.

Key regulatory elements

Regulatory elements coordinate the precise expression of genes in different cell types at the correct developmental stages and in response to changes in external conditions. Generally located within non-coding regions, they can exert their effects through various processes. An important class of element comprises specific regulatory sequences that affect transcription through binding with various proteins, generally known as TFs, including activators and repressors [4]. TFs can bind to cis-regulatory elements located in transcription start sites (TSS) such as promoters, as well as to distal elements including other regulatory regions that can be located thousands of base pairs away from the TSS, such as enhancers, silencers and insulators [12–14]. A representation of these cis-regulatory elements is shown in Figure 2. TFs also ensure the correct positioning of RNA polymerase II (RNAPII) to enable the correct formation of the transcription-initiation complex [15]. These regulatory elements are often located in conserved regions [16]. Key regulatory elements in this class include:

- Promoters: DNA sequences located in the 5’ region of genes that activate transcription via RNAPII. Through the action of various TFs, the RNAPII binds to a consensus sequence, the TATA box, forming the RNAPII transcription-initiation complex [17].
- Enhancers: Short DNA regions that can be bound by DNA-binding proteins called activators and increase gene transcription through their interaction with RNAPII. They can be located thousands of base pairs away from the TSS, but through their interaction with activators, they often form DNA loops that bring them closer to the promoter region [18].
- Silencers: Short DNA sequences that bind to DNA-binding proteins called repressors, causing a decrease in gene transcription by inhibiting other genomic elements such as promoters. Like enhancers, they can be located near the TSS or thousands of base pairs away from it, forming loops using DNA-binding and other proteins to get closer to the promoter regions [19].
- Insulators: These regulatory sequences have a key role in chromatin state regulation, by preventing interactions between chromatin domains. Probably the most well-known is the transcriptional repressor CTCF (CTCF). By binding with target sequences, CTCF can act as an insulator by both blocking interactions between enhancers and promoters and preventing heterochromatin expansion, effectively acting as a chromatin barrier [20].

These functional elements often form clusters that regulate the expression of the same or different genes [21]. As a result, a single loss-of-function mutation in a cluster of enhancers will not necessarily alter gene expression leading to pathogenic effects, due to redundancy between the enhancers in the cluster [22]. However, a gain-of-function mutation is more likely to result in a pathological phenotype due to expanded enhancer activity [22].

Another important class of regulatory elements includes the non-coding RNAs (ncRNAs). They tend to influence regulation post-transcriptionally, by modifying the primary transcript or mature miRNA. This process can involve several mechanisms, including capping, splicing and polyadenylation [14, 23, 24], as well as binding to proteins. For example, ribonucleoproteins are RNA-containing proteins that take part in splicing processes, regulating miRNA maturation [25]. Post-transcriptional regulation can dramatically affect mRNA abundance and can be carried out via different types of ncRNAs, the most well-known of which include the following:

- miRNA: Small, single-stranded RNA molecules (~25 nucleotides) that are involved in gene silencing processes. These small molecules are often found in non-coding intronic regions and are generated by the Dicer enzyme and the RNA-induced silencing complex (RISC). Dicer cuts the miRNA precursor molecule and the mature miRNA is then incorporated into the RISC. This allows the complex to recognize miRNAs complementary to the mature miRNA and cleave them, preventing the miRNA from being translated into protein [26].
- lncRNA: Long non-coding RNA molecules (~200 nucleotides) that are thought to be involved in many types of gene regulation, not only post-transcriptionally. They can interact with TFs to modify their activity, such as those that regulate RNAPII [27]. They also bind mRNA molecules to affect post-transcriptional gene expression [28]. They can also play a role in the regulation of mRNA translation [29] and epigenetic modification by affecting histone methylation [30].
There are other types of ncRNAs that can influence regulation, including enhancer RNAs [31], piwi-interacting RNAs [32] and more [33]; the full details of which are outside the scope of this review.

Clinical genome sequencing for detecting variants in patients

It is now possible to sequence the genome of a patient and detect their variants in matter of hours using next-generation sequencing (NGS) [34]. Initial problems related to technical issues such as sequencing errors still persist but are being ameliorated by technological improvements and advances in bioinformatics techniques [35]. Although tools for prioritizing variants associated with coding regions and predicting their effects on protein structure and function are well established [36], predicting the function of regulatory variants is a relatively new area of research. As the majority of variants in non-coding regions have no obvious effect on disease, and despite multiple notable efforts such as the aforementioned ENCODE project, the characterization of all non-coding elements and how they can affect disease is still far from complete [37]. In the next section, we will describe the state of the art in sequencing platforms for clinical genomics and variant determination, including key methods for pre-processing, alignment and genomic variant calling. This procedure is largely the same for coding and regulatory variants, with some important distinctions that will be discussed.

Current sequencing platforms

There are two main considerations when selecting a sequencing platform for variant determination: (i) coverage per nucleotide: the number of reads that support a certain genomic position, better if the selected technology gives a larger number of sequences per run, and (ii) sequencing error rate: the average proportion of nucleotides not correctly sequenced. The most well-known sequencing platforms are Illumina, Roche 454, PacBio and Oxford Nanopore. Despite recent advances in the latter two platforms, Illumina remains the most popular and well-established sequencing technology for single-nucleotide variant determination. It allows the generation of a large number of short reads, ensuring a good coverage per nucleotide ratio [38], indispensable for the correct determination of this type of variant. Illumina can also be used for targeted gene sequencing [39], which can also be applied to the sequencing of predetermined regulatory regions, and has overtaken Roche 454 for clinical application [35]. PacBio and Oxford Nanopore, which

![Figure 1. Description of the steps necessary to characterize regulatory variants. Following DNA extraction and sequencing of individual samples, a number of computational steps are performed. First, trimming and alignment of the sequences is necessary. Then a file with all the variants is obtained using the variant calling procedure. At this point, these variants must be associated with disease, using GWAS or other experimental designs such as trio analysis. Next the non-coding SNPs can be analysed and annotated putatively based on overlap with genomic functional elements (variant annotation) and their putative effects can be predicted (variant effects prediction). In orange boxes, there are some examples of tools or analysis types that can be used for each step, and in the case of variant annotation and variant effects prediction, we show examples of tools focused on regulatory variants.](image-url)
generate long reads, look set to play a key role in the detection of large regulatory regions in the future, due to their ability to detect structural variants; however, they currently have a relatively low coverage, making them unsuitable for the routine detection of single-nucleotide variants at this moment in time [40, 41]. Therefore, we will focus on Illumina, who claim that their latest sequencing platform, HiSeq V, can sequence one human genome per run in a single day for $1000, with a coverage of 30X [42].

Figure 2. Representation of the effects of cis-regulatory elements: enhancers (A), silencers (B) and insulators (C). In 2A, the enhancer region binds to a protein (activator) that joins to a specific transcription factor binding site (TFBS) in the promoter region, upregulating the target gene. In 2B, the silencer region binds to another protein (repressor) that binds to a specific TFBS in the promoter region, leading to reduced gene expression. Finally, in 2C, the insulator region interacts with the activator protein of an enhancer, blocking its binding to the promoter and inhibiting gene expression. These interactions are highly controlled and dynamic, and modifications to these elements can dysregulate expression and lead to disease.

Read pre-processing and alignment

Pre-processing

Illumina, like most sequencing platforms, produces a FASTQ format data file, consisting of the raw sequences (reads), derived from the patient genome, and their assigned quality scores, represented as a Phred score (Q), which shows the probability that a nucleotide is erroneously sequenced, expressed as $P_{\text{nt}} = -\log(Q)$. These scores should be used to remove
poor-quality reads and adapters and detect other errors produced during the sequencing process [43, 44]. This can be performed using various tools such as Trimomatic [45] or Cutadapt [46]. There are also various suites such as BBTools, which has a range of tools for these processes.

Alignment

Once the reads have been pre-processed, they must be aligned to a reference genome to allow us to identify areas where they differ, indicative of variation in the sequenced genome [47]. Good alignment tools must be both accurate and fast, as they are typically dealing with many millions of reads per sample; moreover, they must discriminate sequencing errors from true variants [48]. Some, such as Bowtie [49], and BWA [50], use the Burrows–Wheeler transform, while others, such as Stampy [51], use hash-based algorithms, which have been shown to give the most accurate results for Illumina sequences in terms of variant calling [48, 52].

These tools generate a Sequence Alignment Map (SAM) file [53], which contains details of the reads and their alignment positions. This is typically then transformed to a BAM (Binary Alignment Map) format, to save space [54]. They include a mapping quality (MAPQ) score, which can be used to decide the best position for a read that matches to the genome in multiple positions. These files are the starting point for the next analysis step: variant calling.

Variant calling

Variant calling tools determine the locations in which a sequenced genome differs from the reference using different statistical methods [52]. They attempt to avoid potential errors that have slipped through the sequencing and alignment processes, using the nucleotide quality scores and minimizing the false-positive variant call rate [55]. Two variant calling software use a Bayesian approach algorithm for determining true variants from NGS data. The first one, the Genome Analysis Toolkit (GATK) [56], is a popular package with customizable modules not only for detecting different types of variants but also for determining whether these variants are likely correctly identified, which makes it one of the most-used tools for variant calling [57]. The second one, FreeBayes, is a framework specially developed for detecting single-nucleotide polymorphisms (SNPs) and short structural variants in haplotypes [58]. Other tools, such as VarScan [59], use statistical approaches to sort and score the alignments and reject those deemed ambiguous. Another tool, LoFreq, is based on a Poisson-binomial distribution and can be used to find rarer variants. In all cases, these tools return a variant call format (VCF) file containing variants, their positions, identifiers and quality scores, among other parameters [60]. Of these variant callers, it is recommended to use FreeBayes when variants with a variant allele frequency less than 0.10 are expected, and if higher than 0.20, the use of GATK is recommended [61].

Strategies for associating variants with disease

Once the VCF has been obtained, it is necessary to determine which variants are related to the disease (pathogenic) and those that have no effect (neutral). A common strategy is to compare allelic and genotypic frequencies between disease sufferers and controls, as is the case in GWAS. These studies are focused on identifying putative genomic variants, such as SNPs, that are associated with disease and population traits [62]. The canonical GWAS procedure compares variants from patients that share the same disease or phenotype against variants from healthy individuals from the same population, used as a control data set. Thus, disease-associated variants can be determined because of their relative frequency [63]. It is important to understand that SNPs associated with a disease are not necessarily causal, instead they may be inherited within the same haplotype block, potentially in linkage disequilibrium (LD) with the casual SNP [64]. This is particularly important when using SNP microarray-derived GWAS data, in which only a representative subset of variants is determined, which means that fine-mapping studies will usually be necessary. In relation to regulatory variants, this is a problem, as a haplotype block may contain several regulatory elements. Multiple testing correction is also critical to reduce the number of spurious associations [65], which can be exacerbated by the structure in the data related to LD [66]. Detecting associations is typically performed using tools such as PLINK, which can use multiple association models such as allelic or genotypic recessive [67]. Other tools include PRESTO [68] and FERMORY, the latter performs its statistical corrections through permutation tests and is optimized for sequencing data [69]. For further information about bioinformatics procedures related to GWAS, we highly recommend the work by Bush and Moore [63]. Higher order associations can also be examined using epistasis-based approaches [70].

Despite their widespread usage and popularity, GWAS have only been able to explain a small proportion of estimated disease heritability [71]. Moreover, the GWAS approach requires a large number of patients to obtain statistical significance in most cases [72]. Other alternative approaches exist for associating variants with disease. These include parent–child trio analysis, which consists of sequencing and comparing the genome of related individuals to distinguish between variants that are inherited from parents and de novo variants [73]. These de novo variants can be putatively associated with disease if the parents do not show the same phenotype as the child. Parent-child trio studies have been shown to decrease the type I error inherent to GWAS, as constructing parent–child relations avoids population stratification [74]. There are several tools to perform parent–child trio analysis using VCF files, such as Trio-SVM [75], which uses a support vector machine (SVM), TrioVis [76], a visualization tool that groups variants according to the Mendelian inheritance model, and trio [74], an R-Bioconductor package able to conduct multiple analyses. Another approach is to perform variant prioritization directly on a given genome, using annotation and other prioritization algorithms [36]. The annotation procedure for non-coding regions will be explained in the next few sections, along with how to prioritize potential regulatory variants in these regions. This remains an important challenge, as we are far from knowing the positions of all regulatory regions across all cell lines, tissues and conditions, and it is a rapidly evolving field.

Projects to detect regulatory elements and their usage to annotate and prioritize non-coding variants

Projects to annotate the non-coding genome

Regulatory variant annotation involves determining the regulatory elements with which variants overlaps. There are many tools available for this process, which use data from multiple resources, including TF binding sites (TFBSs), enhancers, promoters, DNA methylation sites, introns and splicing sites and
others. Several consortia and international projects have been set up to produce such data, with the aim of identifying and characterizing all the regulatory elements in the non-coding genome, using multiple experimental techniques including Chromatin Immunoprecipitation Sequencing (ChIP-Seq) [4], chromosome conformation capture methods [77], DNase I hypersensitivity assays and DNase Sequencing (DNase-Seq) [78] and RNA Sequencing (RNA-Seq) [79]. Further information regarding regulatory sequence identification can be found here [80, 81]. Key international projects for the characterization of regulatory elements within the human genome include the following:

- **ENCODE** (https://www.encodeproject.org/): The ENCylopedia of DNA Elements project combines the efforts of many research groups to create a full catalogue of functional annotations of genomic elements [81, 82]. Through various high-throughput techniques, they have identified regulatory regions in diverse cell lines, providing information on regulatory features such as TFBSs and motifs, TSS, histone marks, DNA methylation sites and open chromatin regions, revealing genomic regulatory mechanisms that were previously unknown. Data can be accessed directly through the ENCODE Web portal or using the online resource University of California Santa Cruz (UCSC) Genome Browser (https://genome.ucsc.edu/) [3].

- **FANTOMS** (http://fantom.gsc.riken.jp/5/): The Functional Annotation of the Mammalian Genome (FANTOM) is an international consortium that undertakes large-scale complementary DNA (cDNA) sequencing projects to map and identify TSS and provide a comprehensive atlas of human gene expression [83], as well as functional annotation of mammalian genomes and expression profiles [84]. Through Cap Analysis of Gene Expression (CAGE), a technology that captures short 5’ ends of mRNA for producing short nucleotide sequences with NGS, they can determine genes, detect TSSs and recognize the activity of regulatory elements such as promoters, enhancers and others [85]. They have identified more than 180,000 promoters and almost 44,000 candidate enhancers in hundreds of primary cells from human and mouse genomes [83]. Data can be accessed through their website and have been used together with ENCODE to identify disease-associated variants within regulatory elements [86].

- **Roadmap Epigenomics Project** (http://www.roaddmapepigenomics.org/): This project aims to create a complete human epigenetics map, using NGS technology for analysing genome marks (DNA methylation, histone modifications, chromatin accessibility and RNA transcripts), in different cell types and tissues [87]. The website offers a genome browser and has a repository to download the latest data sets. Information can be used for identifying and annotating disease-associated variants that affect specific regulatory elements whose expression is different according to cell type, tissue or the stage of development [88].

- **GTEx** (https://www.gtexportal.org/home/): The Genotype-Tissue Expression project encompasses a large community of research groups with the shared aim of demonstrating the relationship between variants and human traits or diseases by analysing changes in gene expression [89]. This project determines expression quantitative trait loci (eQTL) by combining genetic variation with gene expression in post-mortem tissues. It can be used to determine which pathways are affected in disease [90]. They provide an expression atlas for the identification of putative regulatory regions and determining eQTLs associated with disease [89].

Other projects include those attempting to unravel the epigenetic profiles of different human cell types, such as the International Human Epigenome Consortium (IHEC) [91] and BLUEPRINT [92]. We describe key resources in Table 1.

### Regulatory variant annotation tools

Various computational tools use the information generated by these projects to annotate regulatory variants. Such tools usually combine genomic information from multiple projects to determine the regulatory elements close to a query variant. Three of the main annotation tools for this process are described here. In addition, in Table 2, we provide details of, to the best of our knowledge, all freely available tools for this purpose.

- **RegulomeDB** (http://regulomedb.org/): It includes data from resources such as ENCODE and the Roadmap Epigenomics Project for annotating variants within regulatory elements [103], including different regulatory features: TFBSs, chromatin states of different cell types and eQTL data. This information is combined to calculate a score for variant prioritization, useful when annotating GWAS variants associated with disease [104].

- **HaploReg** (http://compbio.mit.edu/HaploReg): It combines information from ENCODE and the RoadMap Epigenomics Project for annotating regulatory variants [106]. To help the user zero in on the causal SNP, the tool combines variants into haplotype blocks, so that SNPs correlated with the query SNP can also be examined. This is achieved using 1000 Genomes Project data, which provides sequence data for many individuals from different populations, for calculating the LD between different variants and producing haplotype blocks. Thus, HaploReg can predict the putative effects of GWAS-derived disease-associated variants and their impact on disease [105].

- **FunciSNP**: The Functional Identification of SNPs is a bioinformatics tool developed in R/Biocomputer that uses genomic data from different resources, such as 1000 Genomes Project and ENCODE, to annotate disease-associated variants [107]. The tool integrates this information to identify SNPs in LD with functional genomic regions. Unlike RegulomeDB and HaploReg, it has no Web service, but is implemented in R.

Other regulatory variant annotation tools include rVarBase [108], FunSeq2 [109], ENlight [110], INFERNO [111], Cepip [112], GEMINI [113], OncoCis [114] and SuRFK [115], and we describe their characteristics, advantages and limitations in Table 2.

### Prediction of the pathogenicity and putative effects of regulatory variants

Many variants overlap regulatory elements in the genome; however, they are not necessarily functionally important. Thus, to predict the potential impact of a putative disease-associated variant, several prediction algorithms have been developed that use positional information of the variant alongside genomic annotations to calculate the probability of this variant to affect regulatory motifs and lead to disease [116]. These algorithms use various classification methods, typically based on machine learning, with the aim of obtaining knowledge from large amounts of data. These include supervised methods, which learn the characteristics that correspond to pathogenic or benign variants using a training data set of known variants. To perform this learning step, functional annotations are used, including regulatory and conserved features from different resources, such as ENCODE. To validate the prediction results, they are compared with a testing data set, also consisting of true pathogenic and non-pathogenic (control) variants. Such variants are typically obtained from the Human Gene Mutation...
Table 1. A selection of databases that contains annotations of regulatory elements

| Name            | Regulatory elements | Database description                                                                 | Reference |
|-----------------|---------------------|--------------------------------------------------------------------------------------|-----------|
| GTRD TFBS       |                     | Stores TFBS information of ChIP-Seq experiments from different resources (including ENCODE) | [93]      |
| TRANSFAC TFBS   |                     | Contains experimental data of eukaryotic TFs, their binding sites, consensus sequences and regulated genes | [94]      |
| JASPAR TFBS     |                     | Includes curated and non-redundant experimentally determined TFBS in different eukaryote organisms | [95]      |
| DENdb Enhancers |                     | Integrates predicted information of enhancers in different cell lines that overlap DNase I HS and TFBS | [96]      |
| Enhancer Atlas  | Enhancers           | Contains annotations of human enhancers from experimental data sets, including histone modifications, TFBS, DNase I HS and additional information using the CAGE technique | [85]      |
| dbSUPER Super enhancers |       | Integrates ChIP-Seq signals of clusters of enhancers in different cell types of human and mouse | [97]      |
| CTCFDB Insulators |                   | Contains information on CTCF binding sites, including experimentally determined and predicted enhancers | [98]      |
| EPD Promoters   |                     | Collects information on promoters recognized by the RNA polymerase II in eukaryotes | [99]      |
| ncRNAcentral ncRNAs |               | Integrates ncRNA information from high-quality resources | [100]     |
| NONCODE IncRNAs |                     | Contains information on ncRNA sequences from various databases | [101]     |

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- **GTRD TFBS:** Stores TFBS information of ChIP-Seq experiments from different resources (including ENCODE).
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- **dbSUPER Super enhancers:** Integrates ChIP-Seq signals of clusters of enhancers in different cell types of human and mouse.
- **CTCFDB Insulators:** Contains information on CTCF binding sites, including experimentally determined and predicted enhancers.
- **EPD Promoters:** Collects information on promoters recognized by the RNA polymerase II in eukaryotes.
- **ncRNAcentral ncRNAs:** Integrates ncRNA information from high-quality resources.
- **NONCODE IncRNAs:** Contains information on ncRNA sequences from various databases.

TF: transcription factor; TFBS: transcription factor binding site; DNase I HS: DNase I hypersensitive site; ncRNA: non-coding RNA; lncRNA: long non-coding RNA.

A recent comparison has demonstrated that FATHMM-MKL and GWAVA outperformed other tools such as CADD and DANN using the same benchmarking data set; however, despite all the advances in regulatory variants prediction, there are still issues related to our incomplete knowledge of regulatory regions and how base-changes can affect them [126]. Other tools described in Table 3 include deltaSVM [116], DeepSEA [127], Eigen [128], GenoCanyon [129], PRVCs [130], ARVIN [131] and DIVAN [132]. In addition, there are specific methods for predicting the impact of somatic and germline regulatory variants associated with...
As we have shown, there is a wide range of tools to predict the potential impact of variants in non-coding regions. However, it is crucial to validate these predictions, in terms of whether the variant overlaps a regulatory element, and how this affects gene expression. This is no easy task and is arguably the most important bottleneck in determining disease-associated regulatory variants. Many cis-regulatory elements (e.g. enhancers and insulators) can be located thousands of base pairs away from the genes that they regulate; moreover, they can regulate multiple genes. Therefore, confirming the impact of regulatory variation requires the use of multiple experimental techniques.

### Experimental methods to identify regulatory variants

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| Name | Uses | Main data sources | Advantages | Limitations | Reference |
|------|------|-------------------|------------|-------------|-----------|
| RegulomeDB | Prioritization of functional variants, using a score based on the number of elements with which the variant overlaps | ENCODE, Roadmap Epigenomics Project | Includes information from numerous functional annotation sources | The scoring system can be difficult to interpret | [103] |
| HaploReg | Annotation of variants in LD, located within or next to regulatory elements | ENCODE, GTEx, Roadmap Epigenomics Project | Allows the identification and mining of causal variants in LD that affect regulatory sites | Functional annotations are not updated periodically | [106] |
| FunciSNP | Identification and prioritization of putative regulatory SNPs | ENCODE, Roadmap Epigenomics Project | Large data queries are fast to perform | A minimum knowledge of R is needed for its use | [107] |
| rVarBase | Annotation of regulatory variants that are involved in transcriptional and post-transcriptional regulation | ENCODE, Roadmap Epigenomics Project | Uses annotations of numerous regulatory features, easy to use, intuitive website | Results summary can be initially confusing, i.e. a SNP can appear annotated with both strong and weak transcription | [108] |
| FunSeq2 | Prioritization of cancer-associated SNVs in non-coding DNA | ENCODE | Can annotate and prioritize variants directly from BED or VCF files and the analysis can be customized | It is specifically designed to annotate cancer-associated variants but not for variants associated with other diseases | [109] |
| ENlight | Annotation of GWAS variants and analysing their putative effects through plot visualization | GWAS, ENCODE, GTEx | Plot system is useful to visually identify causal variants and the analysis can be customized | Functional annotations are not updated periodically | [110] |
| INFERNO | Characterization and prioritization of regulatory variants in different tissues | GTEx, FANTOM5, Roadmap Epigenomics Project | Prioritize variants by calculating an empirical p-value | Large Web queries take a long time to complete | [111] |
| Cepip | Prioritization of gene regulatory variants using tissue-expression data and predicted scores | GTEx, ENCODE, scores from different prediction tools | Integrates the effect of multiple chromatin states to identify and prioritize functional regulatory variants | A minimum knowledge of the command line is needed for its installation and use | [112] |
| GEMINI | Annotation of non-coding variants by integrating chromatin information for different cell types | ENCODE | Incorporates a workflow that automatically annotates variants from VCF or pedigree files | Requires command line use and lacks regulatory features in comparison with some other annotation tools | [113] |
| OncoCis | Prioritization and annotation of cis-regulatory somatic variants in cancer samples | ENCODE, Human Epigenome Atlas, Jaspar, FANTOM5 | The annotation procedure is more rigorous in comparison with other tools for identifying cis-regulatory mutations and it can be applied for identifying cell type-specific variants | It is specifically designed to annotate cancer-associated variants but not for variants associated with other diseases | [114] |
| SuRFR | R package that integrates annotations from different resources to prioritize functional regulatory SNPs | ENCODE, FANTOM5 | Short execution times and higher data confidentiality in comparison with Web-based tools | The user must be familiar with the R programming language | [115] |
Table 3. Summary of the main non-coding variant effect prediction tools

| Name       | Description                                                                 | Type                | Advantages                                                                 | Limitations                                                                                           | Reference |
|------------|------------------------------------------------------------------------------|---------------------|---------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|-----------|
| CADD       | Framework designed to predict the impact of variants in coding and non-coding regions | SVM                 | Precomputes C-scores that can be used for other tools to prioritize variants | Limited performance due to SVM, as this cannot capture non-linear relationships between annotations    | [120]     |
| DANN       | Software that predicts the deleteriousness of genetic variants, using the same data set as CADD with a different algorithm | DNN                 | DNN algorithm can capture non-linear relationships between annotations that SVM cannot | Does not have a website and the user requires Python knowledge to use it                                | [121]     |
| GWAVA      | Tool for predicting the impact of coding and non-coding variants using different annotations to prioritize those that are functional | Random forest       | The random forest algorithm is modified to overcome the class imbalance issue when combining different genomic features | During the execution through the website, parameters to prioritize variants cannot be modified and it only supports a single region as a query | [122]     |
| FATHMM-MKL | Software for predicting the impact of coding and non-coding SNVs using annotations grouped in categories | MK learning         | They convert annotation groups into multiple kernels to perform the evaluation, and it outperforms other tools for non-coding SNV effects prediction | During the execution through the website, parameters to prioritize variants cannot be modified          | [123]     |
| FATHMM-XF  | FATHMM-MKL improvement, with extended features gathered in a single-kernel data set | Supervised machine learning | In terms of accuracy, outperforms other prediction tools, including its predecessor, FATHMM-MKL, for non-coding SNV effect prediction | During the execution through the website, parameters to prioritize variants cannot be modified          | [124]     |
| LINSIGHT   | Tool that determines the probability of negative selection in non-coding regions to predict the impact of variants that overlap them | Linear and probabilistic models | Combines both linear and probabilistic model for functional genomic and evolutionary data, respectively, to identify causal inherited non-coding variants in conservation sites | The user must be familiar with the use of the command line to install and execute the code               | [125]     |
| deltaSVM   | Software that predicts and quantifies the impact of regulatory variants based on regulatory features whose role is cell-dependent | SVM                 | Uses a catalogue of DNase I hypersensitivity sites, histone modifications and TFBSs information to predict the effects of variants within enhancer regions with high accuracy | The user must be familiar with the use of the command line to install and execute the code and lacks genomic features in comparison with other tools | [116]     |
| DeepSEA    | Tool for predicting the functional impact of non-coding variation by evaluating molecular function | Deep learning       | It combines de novo (predicted) information of chromatin effects and conservation sites to prioritize functional variants | Using the default threshold, DeepSEA returns a large amount of information, even for a small search using only a handful of variants | [127]     |
| Eigen      | Software for scoring, prioritising and predicting the impact of putative causal coding and non-coding variants using ENCODE annotations | Unsupervised spectral learning | In comparison with other score calculations, Eigen uses a more refined annotation data set to make its predictions and the unsupervised algorithm deals well with the class imbalance issue | To download and use of the tool, the user has must be familiar with the use of the command line and R | [128]     |
| GenoCanyon | Tool that calculates a prediction score for each nucleotide of a given genomic region, using conservation data and epigenetic information | Unsupervised statistical learning | Predicts impact using conservation sites and molecular features, generating a plot that includes the analysed region and the prediction score for each nucleotide | Large regions cannot be analysed through their Web app and parameters other than those related to the plot cannot be changed | [129]     |

Continued
These include determining the location of different regulatory elements through chromatin association methods and molecular cytogenetic techniques, and correlating variants with changes in gene expression. The genome editing technique, CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-Associated Protein 9), is likely to be of high impact in this field.

Determining regulatory element location

Chromatin association methods

Once a variant has been predicted as pathogenic, multiple experimental techniques can be used to identify whether it overlaps a regulatory element. Chromatin association methods can be used for this purpose, by determining the physical connections between different loci, allowing us to visualize interactions between different areas of the genome; thus, we can explore whether variants affect these interactions. Multiple techniques are available, further described in Table 4, such as Hi-C, which had led to the development of full-genome interaction maps for different cell types and developmental stages [136]. However, although these methods can be used to analyse interactions between loci, they cannot identify interactions between loci and proteins. For this purpose, it is necessary to adapt the chromatin association methods and combine them with other techniques, such as molecular cytogenetic techniques.

Molecular cytogenetic techniques

These include DNA fluorescence in-situ hybridization (FISH) [138] and combined methods like chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) [139], which uses both chromatin immunoprecipitation and chromatin interaction-based techniques with NGS. Such methods are able to detect interactions between proteins and their binding sites with high resolution [140]. FISH has been used to study the three-dimensional organization of the chromosomes. This technique allows the visualization of interactions between loci located far away in individual cells [141] and has several applications. In combination with other chromatin-based techniques, for example with capture Hi-C, it can improve the resolution for detecting physical chromatin interactions and open chromatin conformations in large genomic regions. This has been useful to determine chromatin interactions in different risk loci where regulatory variants have been associated with colorectal cancer [142]. In addition, it can be used in combination with luciferase reporter assays to detect the activity of functional regulatory elements [143]. Moreover, it has recently been demonstrated that modifications of this technique, such as the single molecule FISH protocol (smFISH), can be used for studying transcriptional and post-transcriptional regulatory processes in different animal model tissues [144]. In the case of transcriptional regulation, this technique can quantify various TFBSs and molecules that bind them [145]. For post-transcriptional regulation, smFISH can be used to quantify the abundance and visually detect ncRNA molecules in different cellular compartments [146].

CRISPR-Cas9 method for evaluating variant effects

Genetic editing of specific loci can provide information about chromatin structure and interactions with other loci and proteins. A revolutionary technique for genome edition is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated nuclease 9 (Cas9), CRISPR-Cas9 system, which can be used to mutate genomic regions of interest to study their effects [147]. For example, using this system it has been possible to target mutations to specific regulatory elements in experimental models: within the intronic CCA(T)/6GG (CARG) box in the promoter region that regulates the expression of CNN1 gene (whose loss of function has been associated with ovarian cancer [148]), and to later apply quantitative techniques to measure the functional impact [149]. CRISPR-Cas9 has also been used to delete TF binding motifs in the β-globin locus control regions and investigate their effects [150]. In addition,
that one of these disease-associated variants, rs9349379, was found in the non-coding regions of 6p24, specifically at the EDN1 gene locus [8]. Using expression data and regulatory annotations from the ENCODE and the Roadmap Epigenomics Project database (mainly histone marks), the authors revealed the regulatory role of the annotated 6p24 region as an enhancer involved in vascular cells [8]. Then, they validated the role of rs9349379 in the expression of the endothelin 1 (EDN1) gene and the peptide ET-1 production [40]. The role of EDN1 is essential for correct vascular development because it codes for the peptide ET-1, which is essential for correct vascular development when it is expressed in the endothelial cells. One of these regulatory variants is a SNP located in an enhancer region that regulates the expression of the endothelin 1 (EDN1) gene in aorta, located 600 bp upstream of rs9349379 [8]. The role of EDN1 is essential for correct vascular tissue development because it codes for the peptide ET-1, which regulates the vascular tone of smooth muscle [152]. To validate the regulatory role of the annotated 6p24 region as an enhancer of EDN1, they performed a targeted deletion in this region using the CRISPR-Cas9 genome editing method, showing an increase in the expression of EDN1 gene and the peptide ET-1 production in vascular cells [8]. Then, they validated the role of rs9349379 variant using CRISPR-Cas9 to generate different allelic series of this single nucleotide, analysing and quantifying the effects of this variant with RNA-Seq. In addition, they measured the three-dimensional contact between EDN1 and rs9349379 using chromatin association methods [8].

### Regulatory variants associated with disease: case studies

Research of variants associated with disease in non-coding regions often exploits existing GWAS data to prioritize variants and annotate the genomic regions to which they map, followed by experimental validation of their putative effects. We will now provide some examples where variants in non-coding regions have been found to be involved in complex diseases. These diseases typically involve combinations of different factors, including environmental, genetics and lifestyle; as such, it is likely that regulatory variants will play an important role, as their influence is often context-dependent.

#### Coronary artery disease

This pathology affects oxygen flood to the heart due to narrowing of blood vessels, leading to chest angina and cardiac arrest. Common causes include high levels of cholesterol in blood, high blood pressure, insulin resistance, sedentary habits, smoking and alcoholism. Through the use of GWAS, risk variants associated with five different vascular diseases, including CAD, were found in the non-coding regions of 6p24, specifically at the PHACTR1 gene locus [8]. Using expression data and regulatory annotations from the ENCODE and the Roadmap Epigenomics Project database (mainly histone marks), the authors revealed that one of these disease-associated variants, rs9349379, was located in an enhancer region that regulates the expression of the endothelin 1 (EDN1) gene in aorta, located 600 bp upstream of rs9349379 [8]. The role of EDN1 is essential for correct vascular tissue development because it codes for the peptide ET-1, which regulates the vascular tone of smooth muscle [152]. To validate the regulatory role of the annotated 6p24 region as an enhancer of EDN1, they performed a targeted deletion in this region using the CRISPR-Cas9 genome editing method, showing an increase in the expression of EDN1 gene and the peptide ET-1 production in vascular cells [8]. Then, they validated the role of rs9349379 variant using CRISPR-Cas9 to generate different allelic series of this single nucleotide, analysing and quantifying the effects of this variant with RNA-Seq. In addition, they measured the three-dimensional contact between EDN1 and rs9349379 using chromatin association methods [8].

#### Crohn’s disease

CD is a complex disorder that mainly affects the lower digestive system [153]. GWAS have found a large proportion of non-coding regions to be associated with the disease [154]. These variants are thought to affect the correct regulation of genes expressed in various tissues, including liver, brain and several immune system cells. One of these regulatory variants is a SNP located within the intronic region of the FOXO3A gene, which has been indirectly associated with CD and other diseases by affecting FOXO3A regulation [9]. In this study, two groups of patients were established accordingly to whether they showed aggressive or indolent CD effects, respectively. Then, they searched for variants that affected the genes involved in two pathways related to this disease, including their flanking regions, with the aim of finding which SNPs differentiated...
each group. With this information, they performed a GWAS re-analysis, and determined a non-coding SNP (rs12121067) within the intronic region of the FOXA3A gene, one allele of which was related to the indolent group of patients with CD, and the other allele to the aggressive group. Finally, they performed an experimental validation to confirm the association between this variant and both groups of patients, using ChiP-qPCR and luciferase assays [9].

Schizophrenia

Schizophrenia is highly complex in terms of genetics, and GWAS studies have revealed that more than a hundred loci are associated with this disease, containing thousands of non-coding risk variants [155]. Several GWAS have been performed for this disease, and most existing studies attempt to confirm the causality of the significantly associated variants to explain their mechanism of action. Such is the case for SNP rs1625579, located in the locus of the MIR137 gene that has been associated with neuropsychiatric disorders, including schizophrenia [10]. Using genotype data from the HapMap project, they found another variant, rs2660304, in strong LD with rs1625579. This rs2660304 SNP affects the internal promoter of the MIR137 gene, reducing its expression. The association of the variant with the activity of the promoter was confirmed by luciferase assays and its regulatory role was determined computationally using the annotation tool HaploReg [10].

Cancer

There is increasing interest in the study of regulatory variants in relation to cancer, both in terms of inherited and somatic mutations [156]. For example, different types of cancer have been associated with variants in the promoter region of the telomerase reverse transcriptase TERT gene [157–159]. In another example, overexpression of MDM2 was found to be associated with the progression of multiple cancers, related to the SNP rs2279744, which affects the gene promoter in both hereditary and de novo cancer [11]. This variant produces an increase in the affinity for the TF Sp1, inhibiting the p53 pathway. To confirm this finding, computational analysis was performed to predict whether this SNP could alter affinity for other TFs. A possible alteration of the E2F1 binding site was predicted due to this SNP, which led to the study of the E2F1-mediated alteration of the transcriptional activity of the MDM2 promoter. To confirm the relationship between rs2279744 and the MDM2 promoter, they performed experimental validation using ChiP-qPCR, luciferase assays and gene silencing assays [11].

Future directions

Recent technological advances, both experimental and computational, combined with the establishment of important projects for data generation and cataloguing, have made it more achievable than ever to find variants in the non-coding regions of the genome associated with disease, map these variants to regulatory elements and predict pathogenicity and validate these variants by investigating how they affect chromosome structure, protein binding and gene expression.

Most of these advances have taken place in the past few years, and they are likely to continue advancing in the near future. Concurrently, we expect new techniques for regulatory element and discovery to emerge. Sequencing technology developments are also likely to play a key role. Although Illumina is currently the most widely used sequencing platform for variant determination, it still has some obstacles to overcome for daily clinical use; nevertheless, we believe that sequencing of patients will become routine in the next decade or so. These data must be kept, alongside detailed phenotypic information on the patient and their symptoms. This influx of data will accelerate the discovery of disease-associated regulatory variants, most of which are likely to be in non-coding regions of the genome. It is therefore important that we complement the expected influx of genetic and phenotypic data by expanding projects to map regulatory regions, as well as eQTL-related projects such as GTEx, to investigate the effects of variants on gene expression. This way we can find not only associations but also start to piece together the regulatory processes involved. Of course, as we have made clear, validation is still the major hurdle in terms of definitively proving the mechanistic link between a variant and its effects; however, we also believe that the parallel development of CRISPR-Cas9-based technologies, alongside molecular techniques to investigate chromatin–protein interactions will help us unravel the full details of how variants cause diseases. Doing so is unquestionably the first step towards developing personalized therapies for patients who suffer from them.

Key Points

- Most investigation of disease-associated variants has been of those that affect the structure and function of proteins. However, increasing importance is now being given to variants that affect expression levels. These regulatory variants usually overlap with regulatory elements.
- We briefly describe the process of obtaining variants from sequencing data, as well as the bioinformatics procedures involved, dealing with the most widely used platforms and software.
- We present important international projects aimed at characterizing regulatory elements throughout the non-coding regions of the genome, such as the GTEx project and ENCODE.
- We describe the key bioinformatics tools for the annotation of regulatory variants and the prediction of their possible impact to classify them as neutral or pathogenic, using a variety of machine learning methods, and how they can be validated, using various experimental approaches.
- Regulatory variants have been determined that affect the expression levels of different genes that give rise to disease. Here we present examples of such variants for complex diseases, including schizophrenia, certain types of cancer, CD and CAD.

Funding

This work was supported by the Spanish Ministry of Economy and Competitiveness with European Regional Development Fund (SAF2016-78041-C2-1-R), the Andalusian Government with European Regional Development Fund (CTS-486) and the Institute of Health Carlos III of the Ministry of Economy and Competitiveness (grants cofunded by European Regional Development Fund, ERDF: PI12/00900, PI12/02247, PI12/02481, PI12/02529, Red de Reacciones Adversas a Alérgenos y Farmacos RD12/0013/0001). The CIBERER is an initiative from the Institute of Health Carlos. 
III. Elena Rojano is a researcher from the Plan de Formacion de Personal Investigador (FP) supported by the Andalusian Government. James Richard Perkins is a researcher from the Sara Borrell Program (CD14/00242) of the Carlos III National Health Institute, Spanish Ministry of Economy and Competitiveness (grants cofunded by European Social Fund [ESF]: CP14/00034 and CP15/00103, respectively).

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