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# Active enhancers: recent research advances and insights into disease

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**Abstract**
Precise regulation of gene expression is crucial to development. Enhancers, the core of gene regulation, determine the spatiotemporal pattern of gene transcription. Since many disease-associated mutations are characterized in enhancers, the research on enhancer will provide clues to precise medicine. Rapid advances in high-throughput sequencing technology facilitate the characterization of enhancers at genome wide, but understanding the functional mechanisms of enhancers remains challenging. Herein, we provide a panorama of enhancer characteristics, including epigenetic modifications, enhancer transcripts, and enhancer-promoter interaction patterns. Furthermore, we outline the applications of high-throughput sequencing technology and functional genomics methods in enhancer research. Finally, we discuss the role of enhancers in human disease and their potential as targets for disease prevention and treatment strategies.

**Keywords** Enhancer, Transcriptional regulation, Multi-omics, Gene editing, Precision medicine

## Introduction

Individual development is an intricate process involving elaborate transcriptional regulations. During development, the identity and function of cells are determined by spatiotemporal gene expression. Transcriptional regulation is pivotal in the orchestration of gene expression, and it controls many intricate and nuanced regulatory processes, such as chromatin remodeling, DNA methylation, histone modification, and enhancer-promoter loops [1–3]. Dysregulation of transcription contributes to a variety of human diseases. Studies on the transcriptional regulatory mechanisms will provide new clues to disease prognosis, diagnosis, and therapies.

Enhancers, as a class of non-coding DNA regulatory elements, are composed of clusters of transcription factor (TF) binding sites and specifically regulate the expression of their target genes through enhancer-promoter interactions. Whole genome sequencing methods have shown that more than two million enhancers have been identified in the human genome [4]. Importantly, active enhancers are characterized by specific histone modification and are tissue specific, making enhancers widely recognized as the core mechanism in the regulation of gene expression and transcription [5–9]. The majority of cancer-associated single nucleotide polymorphisms (SNPs) are located in enhancer regions, and enhancer dysfunction can lead to aberrant gene expression profiles and promote the activation of oncogenes [10–12]. An in-depth study of enhancer will provide novel insights into

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the association between gene expression and biological processes, such as development and the onset of diseases.

Enhancers greatly outnumber the list of protein-coding genes in mammalian genomes, and each enhancer can regulate multiple genes [13]. Uncovering the functional roles of enhancers in disease and development, as well as their regulatory mechanisms on target genes, remains a major challenge in the field. Thanks to the remarkable advancements in high-throughput sequencing and CRISPR/Cas9 gene editing technology, significant progress has been made in the study of enhancers in recent years. In this review, we first discuss the characteristics of enhancers and the models of enhancer-promoter interactions. We then summarize methodologies and techniques used to identify potential enhancers and validate their function. Finally, we discuss how enhancer abnormalities are linked to common human diseases and outline open questions and challenges for enhancers in diagnosis and therapeutic intervention. This review highlights the pivotal role of enhancers in governing transcriptional regulation and diseases, illuminating our understanding in the genetic elements-enhancers and mapping out the trajectories for future research endeavors.

## Patterns of enhancer activation, transcription, and interaction with promoters

How enhancers are epigenetically activated to modulate transcriptional dynamics during development has been extensively studied. Enhancers, as key transcriptional units, pave significant pathways for understanding the transcriptional mechanisms. In addition, the interaction between enhancers and promoters creates an efficient regulatory pattern for transcription.

### Enhancer-specific modifications

Since the first enhancer was discovered in simian vacuolar virus 40 (SV40) in 1981 [14], enhancers have received increasing attention in the following decades. Their properties, structures and regulatory mechanisms are gradually being elucidated. Enhancers possess specific motifs that enable them to recognize and bind to TFs. Their high sensitivity to deoxyribonuclease I (DNase I) facilitates the selective binding of these TFs and cofactors, thereby promoting transcriptional activation [13, 15]. In addition, many coactivator proteins recruited to enhancers have histone acetyltransferase activity, which catalyze histone acetylation. Notably, the histone acetyltransferase p300 and CREB-binding protein (CBP) are known to catalyze the acetylation of histone H3 lysine 27 (H3K27ac). The deposition of H3K27ac marks on histones at active enhancers indicates that this modification is a characteristic feature of enhancers engaged in transcriptional activation [16, 17]. In addition to H3K27ac, enhancers possess histone modification signatures the monomethylation of histone H3 lysine 4 (H3K4me1) and the trimethylation of histone H3 lysine 4 (H3K4me3), as well [18]. Thus, the signatures of histone modifications are intimately associated with the activity state of enhancers. In an inactive state, enhancers reside within a closed chromatin conformation, without transcription factor binding or characteristic histone modifications (Fig. 1A). Enhancers characterized solely by the activating mark H3K4me1 are typically in a primed state, ready for activation (Fig. 1B), whereas enhancers bear both the activating mark H3K4me1 and the repressive mark H3K27me3 are often found in a poised state (Fig. 1C). In an active state, enhancers are enriched with both the activating H3K27ac and H3K4me1 modifications, facilitating the binding of TFs and co-activators (Fig. 1D). This interaction establishes enhancer-promoter (E-P) loops, thereby driving transcription [16, 19, 20].

### Enhancer transcription

Earlier studies found that the hypersensitive site 2 (HS2), located 55 kb upstream of the β-globin locus, possesses prominent enhancer activity and can produce transcripts [21, 22]. By 2010, it was found that enhancers commonly initiate transcription to produce enhancer RNAs (eRNAs) [23, 24]. Unlike mRNA, most eRNAs are short (approximately 0.5-5 kb), bidirectionally transcribed, and do not encode protein [7, 25]. Specifically, due to the degradation effect of exosomes on the 3' end of eRNAs, the tail-adding process akin to mRNA is impeded, resulting in the majority of eRNAs being non-polyadenylated [26]. Recent studies have shown that the eRNA expression levels typically correlate with enhancer activity. Therefore, eRNA expression can be used as a reliable marker for the identification of active enhancers [27–29]. Furthermore, multiple studies have shown that eRNAs are essential for enhancer function. Disruption of the eRNA expression levels can significantly affect the ability of enhancers to modulate transcription [4, 30–35]. In addition, aberrant expression or dysfunction of eRNA is closely related to the occurrence and development of various diseases [31, 36]. The discovery of eRNA is of great importance for the identification of enhancers and the in-depth study of functional mechanisms, while how eRNA affects enhancer function and the impact of eRNA on disease warrant further inquiry.

### Enhancer-promoter interaction

During development, enhancer-promoter interactions (EPI) in the genome mediate on/off patterns of gene transcription in specific cell types. EPI act collaboratively to achieve precise spatiotemporal expression of genes. Although enhancers are often located at considerable distances from promoters within the genome, they paradoxically play a crucial role in long-range gene regulation.

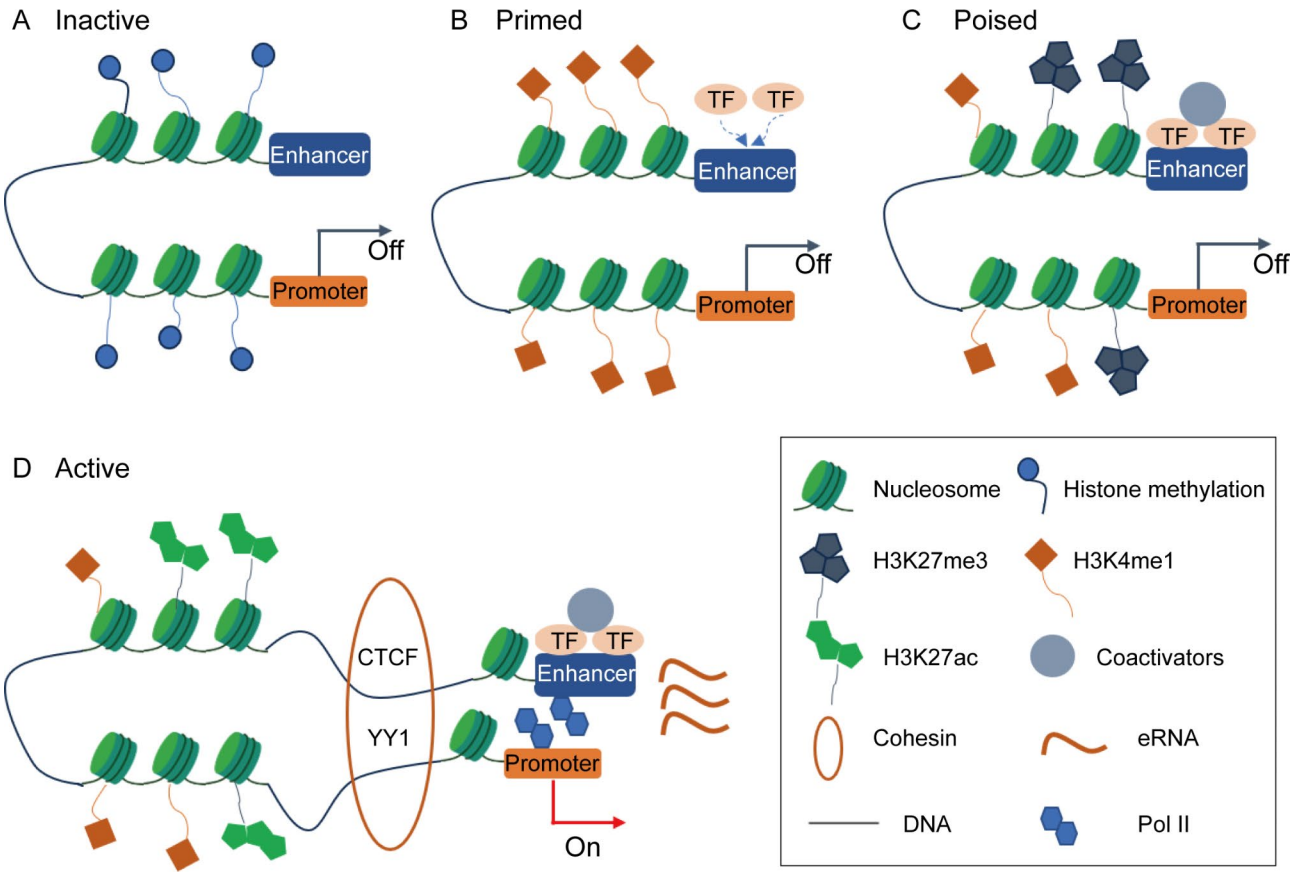


**Fig. 1** Typical characteristics of enhancers in different states. **A** Inactive enhancers are in closed chromatin without transcription factors binding or characteristic histone modifications. **B** Primed enhancers solely have H3K4me1 marks, ready for activation. **C** Poised enhancers are marked by the activating H3K4me1 and the repressive H3K27me3. **D** Active enhancers have H3K4me1 and H3K27ac modifications, bind TFs, recruit coactivators, and form E-P loops

The underlying mechanisms still require further investigation. High-throughput chromosome conformation capture (Hi-C) data indicate that mammalian genomes are organized into numerous higher order structural domains called topologically associated domains (TADs) [37, 38]. The presence of TADs can compress the spatial distance between enhancers and promoters, promoting enhancer-promoter interactions (Fig. 2A) [39, 40]. The boundaries of TADs in mammals are enriched in CTCF and cohesin binding [41, 42], and depletion of CTCF protein or cohesin leads to the disruption of TAD structures [43, 44]. Evidence suggests that TADs boundaries can restrict the interaction of an enhancer with multiple promoters within the same TAD, as deletion of these boundaries can lead to aberrant enhancer-promoter communications [45]. Taken together, most enhancer-promoter interactions occur within TADs, and the high order topological structure of TADs increase the proximity of long-range enhancer-promoter interactions [46].

Even TADs are considered as functional units of transcriptional regulation, enhancers and target promoters within TADs may still be far apart. The precise mechanism by which distal enhancers communicate regulatory information to target promoters over long distances remains an unresolved issue in the field. In recent years, four major enhancer-promoter communication models have been proposed, including the tracking model (Fig. 2B) [47, 48], the linking model (Fig. 2C) [47, 49], the looping model (Fig. 2D) [50, 51], and the looping-tracking/linking model (Fig. 2E) [47]. The tracking model is based on RNA polymerase II (Pol II) binding to active enhancers, which relies on the motor force of Pol II elongation to move along chromatin until the enhancer comes into contact with the promoter [47]. The linking model proposed that protein oligomerization links distal enhancers to target promoters, thereby promoting transcription [52]. The looping model is based on the enhancer-bound protein complex interacts with the transcription factors in the promoter region, prompting the enhancer to bend toward the promoter region, ultimately forming an E-P loop [53]. The looping tracking/linking model is a combination of the above three models, i.e., the E-P loop can bring the enhancer close to the promoter and further shorten the distance by tracking or linking [47]. The chromatin looping model, which brings enhancer and promoter elements into proximity through

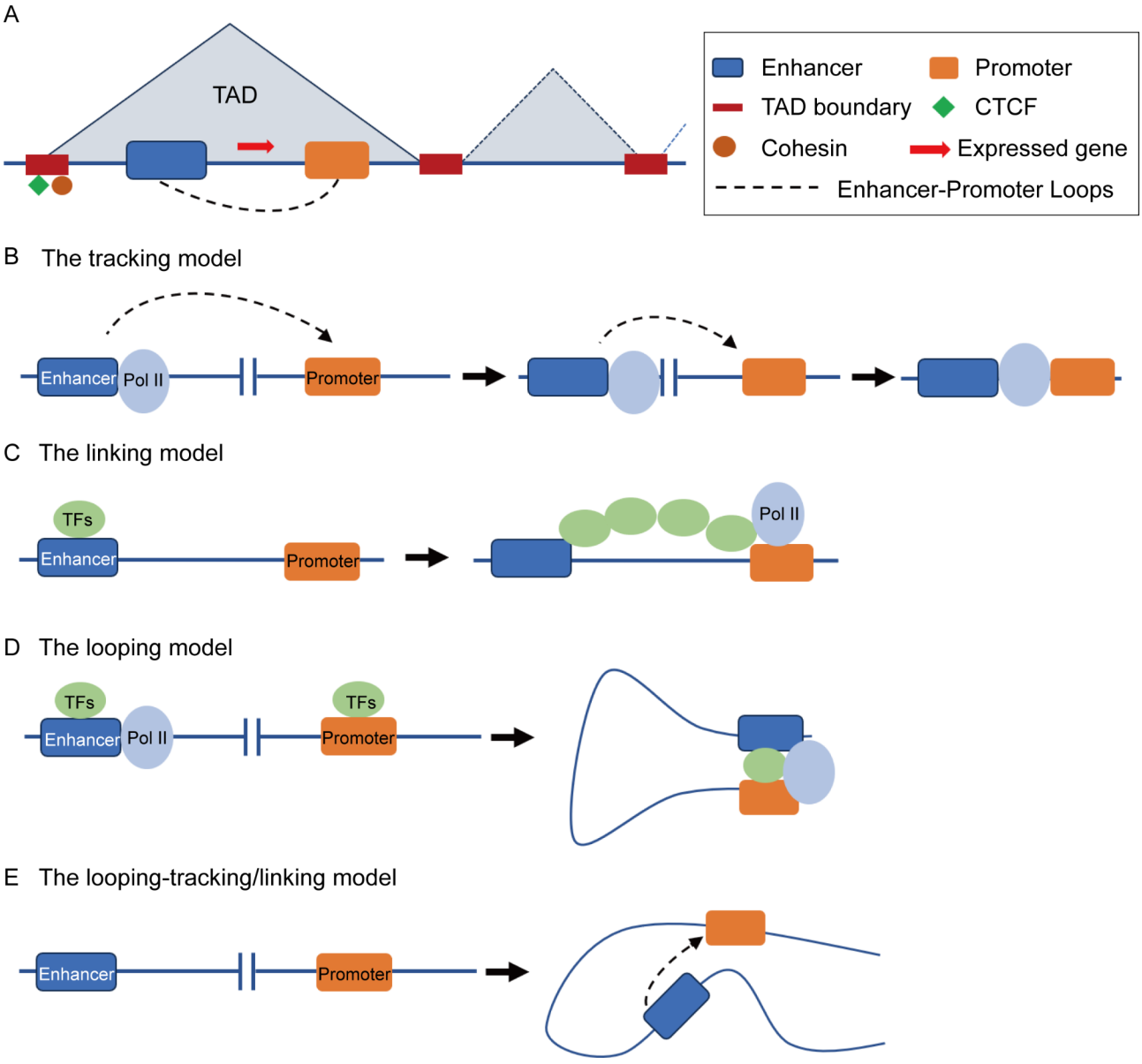


**Fig. 2** 3D chromatin architecture and communication models of enhancers and promoters. **A** TADs are delineated by boundaries that are enriched with the architectural protein CTCF and the cohesin complex. The presence of TADs enhances the proximity and interaction between enhancers and promoters, thereby facilitating more efficient enhancer-promoter communications. **B** Pol II binds to enhancers and moves along chromatin to promoters. **C** An oligomeric bridge is formed by the interaction of several TFs and related proteins connecting enhancers and promoters. **D** TFs and related proteins that bind to enhancers interact with proteins in the promoter region and bend toward promoters. **E** Enhancers interact with distant promoters. Long-range loops bring enhancers close to promoters, and tracking or linking models further shorten the distance between enhancers and promoters

physical interaction, has been widely accepted over the past decade [54]. Although current research cannot fully explain the deeper details of transcriptional regulation mediated by chromatin loops, several chromatin structural proteins, such as CTCF, YY1, cohesin, mediator, and integrator proteins, play a crucial role in the formation of chromatin loops during this process [43, 55–60]. Chromatin conformation capture technologies and their derivatives have been employed to explore the higher order organization of chromatin, providing more feasible methods to study enhancer-promoter interaction patterns from the perspective of 3D chromatin structure [61–63].

How to accurately define the regulatory elements enhancers and promoters? Classically, enhancers and promoters have been defined as regulatory elements with different functions that can be clearly distinguished based on their position and length. The continuous development of high-throughput sequencing technology has enabled large-scale and in-depth studies of enhancers and

promoters in the genome, and it has been gradually discovered that enhancers and promoters have many similar characteristics, such as chromatin structure, sequence features, and regulatory functions [25, 64–66]. Enhancers can have nucleosome-depleted region (NDR) like promoters and initiate transcription at the edge of the NDR [67, 68]. Furthermore, enhancers can act as promoters and, conversely, promoters can also act as enhancers. For example, in human embryonic stem cells, 17 out of 45 active enhancers associated with the *POU5F1* gene are annotated as promoters [69]. Among the 20,709 promoters detected in the human chronic myeloid leukemia K562 cell line, 3% of the promoters exhibited strong enhancer activity, and these promoters with enhancer activity are called "Epromoters" [70]. The promoter of *INS* can interact with the promoter of *SYT8* to regulate the expression of *INS*, indicating that the promoter functions as an enhancer [71]. The complexity of transcriptional regulation is worthy of further exploration.

## Tools to study enhancers

The dynamic alterations within non-coding genomic regions, which significantly impact biological development and disease processes, are increasingly gaining recognition. The difficulties in the study of non-coding regions, including enhancers, are mainly caused by high-level structures, and their function may require the interaction of multiple RNA molecules, structural proteins, and more than two regulatory elements. Currently, more novel methods have been developed to annotate enhancers at the genome-wide scale based on the typical characteristics of enhancers described above. The integration of these methods allows large-scale screening of enhancers in the genome, annotation of functional enhancers, and elucidation of regulatory mechanisms, thereby enabling multidimensional and systematic studies of enhancers.

### Active enhancer identification at genome wide

The rapid development of high-throughput sequencing technology combined with multi-omics data analysis provides powerful methods for enhancer identification.

#### *ChIP-seq and its derivatives*

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is broadly used to study chromatin regulation and to identify the genome-wide binding sites of specific TFs, which can reflect DNA-protein interactions. ChIP-seq generally involves cross-linking cells or tissues with formaldehyde, followed by fragmentation of the chromatin by enzymatic digestion and subsequent enrichment and purification of target protein-bound DNA fragments using anti-histone modification or transcription factor-specific antibodies to construct a library. The DNA library is then subjected to high-throughput sequencing and computational analysis [72, 73]. According to the histone modifications associated with enhancer activity (e.g. H3K27ac and H3K4me1), ChIP-seq can screen putative enhancers across the genome and identify different enhancer states. For example, an enhancer specifically activated in osteocytes was identified to promote osteoblast differentiation by regulating *RANKL* expression, using ChIP-seq data of H3K27ac [74]. In addition, from the ChIP-seq data of TFs potentially binding with a specific enhancer, the potential target genes of the enhancer can be predicted or validated, thus providing clues for the regulatory mechanism of the enhancer [75, 76].

Cleavage under targets and release using nuclease (CUT&RUN) and cleavage under targets and tagmentation (CUT&Tag) are techniques derived from ChIP-seq that compensate for the drawbacks of ChIP-seq, which are time consuming, labor intensive, and require large sample sizes (Table 1). CUT&RUN and CUT&Tag does not require sonication or formaldehyde crosslinking of chromatin and allow DNA cleavage by pA-MNase or pA-Tn5. Like ChIP-seq, CUT&RUN is well suited for studying histone modifications, transcription factors and cofactors and their association with chromatin, but

**Table 1** Comparison of ChIP-seq, CUT & RUN and CUT&Tag methods

| Features | ChIP-seq | CUT&RUN | CUT&Tag |
|---|---|---|---|
| Formaldehyde crosslinking | Yes | No | No |
| Fragmentation | Sonication breaking | MNase enzyme | Tn5 transposases |
| Counting the starting cells | Million | Hundred thousand | As little as dozens |
| The sequencing depth | High (~20 M-100 M reads) | Medium (~8 M reads) | Low (3 M-5 M reads) |
| Library construction | 3 days | 2 days | 1.5 days |
| The scope of application | Widely used for histone modifications, epigenetic modifications, and transcriptional regulation | Widely used for histone modifications, epigenetic modifications, and transcriptional regulation | Unsuitable for proteins that bind weakly to chromatin |
| Advantages | Widely applicable | Better repeatability, low background | High signal-to-noise ratio, fast cycle time, good repeatability |

CUT&Tag is not ideal for proteins that bind weakly to chromatin [72, 73, 77, 78].

#### *ATAC-seq*

Assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq) is a technique to study chromatin accessibility. It primarily uses DNA transposases following by high-throughput sequencing to provide information on open chromatin regions. Compared to DNase-seq, micrococcal nuclease sequencing (MNase-seq) and formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq), ATAC-seq has obvious advantages, such as no use of restriction enzyme, less sample consumption, faster and more sensitive [79–83]. ATAC-seq enables nucleosome localization, chromatin opening mapping, transcription factor identification, and potential enhancer screening. The mesenchymal-subtype GC (Mes-GC) specific enhancer landscapes were depicted by integrating ATAC-seq with ChIP-seq data [84].

#### *RNA-seq and its derivatives*

RNA sequencing (RNA-seq) studies RNA expression levels, transcriptional structure, and splicing patterns at the transcriptional level. Given that active enhancers could also produce eRNAs, RNA-seq-based assays have been used to identify active enhancers by detecting the expression levels of eRNAs [85]. It is worth noting that the expression level of eRNAs is relatively low, the detection of eRNAs may require a deeper sequencing depth. To address this problem, a highly sensitive cap analysis of gene expression (CAGE) technique was developed to capture the 5′ end of transcribed and capped RNAs only. Using CAGE technology, researchers have identified more than 60,000 enhancers in 180 types of human cells, greatly advancing our understanding of enhancers [86, 87]. As a derivative of RNA-seq, global run-on sequencing (GRO-seq) can capture nascent RNAs binding to actively transcribing RNA polymerase and has been systematically used to detect eRNA levels to annotate active enhancers [88, 89]. To identify active enhancers, Wang et al. analysed GRO-seq data from seven cancer cell lines and further investigated the function of these enhancers in different cancer types [90].

#### *Massively parallel reporter assay*

For the traditional reporter assay, a putative regulatory element is inserted into a vector upstream of a reporter (e.g., luciferase), then the vectors are transiently transfected into cells, finally the function of the regulatory element is determined by assessing the activity of the reporter [91]. As a derivative of traditional reporter assay, Massively Parallel Reporter Assay (MPRA) is performed by measuring the activity of hundreds of thousands of enhancers simultaneously, as each enhancer is inserted upstream of a minimal promoter and a reporter gene with a unique sequence barcode. Consequently, numerous potential enhancers can be evaluated in a single experiment by using high-throughput sequencing [92]. To determine whether lncRNA loci might contain enhancer activity, an MPRA library containing six lncRNA loci were transfected into C2C12 cells, followed by RNA-seq, and three lncRNA loci display significant enhancer activity within their gene bodies [93]. An alternative MPRA approach, self-transcribing active regulatory region sequencing (STARR-seq), has also been developed [94]. Unlike MPRAs, which rely on barcode sequences, STARR-seq inserts small genomic DNA fragments into the 3' UTR position of the reporter gene. Following the transfection of vectors into host cells, deep sequencing is then employed to directly assess the reporter gene transcripts. This assay enables the screening of fragments with enhancer activity, as active enhancers are capable of driving the self-transcription of the reporter genes [95]. STARR-seq enables the parallel and direct quantitative assessment of enhancer activity for millions of candidate sequences of arbitrary length and origin, thereby establishing a genome-wide enhancer map [96].

#### *Hi-C and its derivatives*

Enhancers are spatially proximate to target promoters through chromatin loops, and the corresponding regulatory relationship between enhancers and promoters has always been a focus of research. Hi-C is widely used to study the three-dimensional (3D) architecture of chromosomes by applying special chemical modifications to DNA and then sequencing the modified DNA to determine the interactions between different regions of the genome, which can be used to discover enhancer-promoter connections [97, 98]. Chromatin interaction analysis by paired-end tag sequencing (ChIA-PET), in situ Hi-C followed by chromatin immunoprecipitation (HiChIP) and Hi-C coupled chromatin cleavage and tagmentation (HiCuT) technologies have been developed to combine Hi-C and ChIP/CUT&Tag technologies, i.e. Hi-C part of enzymatic cleavage, biotin labeling and ligation experiments are performed first, then specific antibodies are used to perform ChIP or CUT&Tag experiments, and libraries are constructed for high throughput sequencing subsequently [98–100]. Hi-C and the derived technologies capture chromatin interactions at genome wide [101]. Potential target genes of enhancers can be predicted from EPIs, as the presence of TADs can bring distal enhancers into the close proximity of target gene promoters, thereby regulating gene expression [102–105].

It is presumed that there are millions of enhancers in mammalian genomes. The aforementioned assays have

facilitated the identification of putative enhancers associated with specific chromatin signatures and have enabled high-throughput, quantitative assessment of enhancer activity. As most enhancers are cell type-specific and physiologically relevant, active enhancers cannot be efficiently screened out using a single technique. In-depth exploration of enhancer research requires continuous development of sequencing technology and effective integration of multi-omics analysis (Table 2). For example, one study comprehensively identified and characterized enhancers by combining biochemical annotation, MPRAs, and CRISPR techniques [72]. In addition, further exploration is required to dissect the function of active enhancer, such as its downstream targets and the transcription factors involved in.

## Methods for enhancer characterization and regulatory mechanisms study

Enhancers are crucial for the precise regulation of gene expression. The characterization of functional enhancers in the context of disease and the elucidation of the underlying regulatory mechanisms are central to the enhancer research. Recently, a number of tools have been developed and provide unprecedented opportunities for the elucidation of enhancer function and regulatory mechanisms further.

### *CRISPR/Cas9*

CRISPR technology has evolved rapidly over the past decade. CRISPR was discovered in *E. coli* as early as 1987, and until 2012, the CRISPR/Cas9 system was revealed to be a powerful gene editing tool by designing single guide RNA (sgRNA) that target specific regions in the genome [106, 107]. Cas9 protein is an endonuclease with two domains (RuvC and HNH) that can cleave double-stranded DNA. The fusion of Cas9 and sgRNA forms the Cas9 ribonucleoprotein (RNP), which is directed to form DNA double-strand breaks (DSBs) in the target region of the genome, and then the cells repair the DSBs by the non-homologous end joining (NHEJ) or homologous recombination repair (HDR), ultimately the knockout of the target region will be achieved [108–110]. Cas9 can be directed to the enhancer region to delete putative enhancer sequences, which is the current standard for the functional validation of enhancers and the screening of potential target genes (Fig. 3A).

### *CRISPR activation and CRISPR interference*

In addition to the use of Cas9 for enhancer deletion, variant forms of Cas9 can be used for enhancer activation or repression. Dead Cas9 (dCas9), which is generated from nuclease-activated Cas9, can be fused to activation effectors (such as VP64 and p65 proteins) or inhibition effectors (such as KRAB and LSD1 proteins) for the activation (CRISPRa) or repression (CRISPRi) of enhancers (Fig. 3B and C). The dCas9 protein does not cleave DNA but instead recruits effector domains to specific genomic loci under the guidance of sgRNA to achieve gene modification. Importantly, this modification is distinct from Cas9 in that it is reversible [111].

CRISPRa was first applied to promoters by fusing dCas9 with the transcriptional activation domain VP64 or p65 protein, thereby activating endogenous gene expression [112, 113]. Since the activating capacity of

**Table 2** Common tools to screen and study enhancers

| Type | Method | Description | References |
|---|---|---|---|
| Chromatin features and histone modifications | ChIP-seq, CUT&RUN, CUT&Tag | Can be used to screen putative enhancers in the genome-wide and identify active enhancers. Study chromatin regulation and identify the genome-wide binding sites of specific TFs. CUT&RUN and CUT&Tag, as upgrades of ChIP, requires few cells, CUT&Tag is for single cell | [72, 73, 77, 78] |
| Chromatin accessibility | ATAC-seq, DNase-seq, MNase-seq, FAIRE-seq | Genome-wide annotation of regulatory regions based on chromatin accessibility for screening of potential enhancers and transcription factors. MNase-seq is a method that reflects chromatin accessibility indirectly, and the other three methods reflect chromatin accessibility directly | [83] |
| Transcriptional assays | RNA-seq, CAGE, GRO-seq | Annotation of active enhancers by eRNA expression levels generated by enhancer transcription. CAGE and GRO-seq methods improved the sequencing sensitivity | [85, 87, 89] |
| Massively parallel reporter assays | MPRA, STARR-seq | Can be used to readout enhancer activity on a large scale and understand the functional sequence of enhancers in depth | [92, 95] |
| Chromosome conformation assays | Hi-C, ChIA-PET, HiChIP | Can be widely used to explore enhancer-promoter connections from the 3D architecture of chromosomes and identify target genes of enhancers | [98–100] |
| CRISPR system | CRISPR screens, CRISPR/Cas9, CRISPRa, CRISPRi | A high-throughput tool for reading out functional enhancers using CRISPR screens. Functional annotation of potential enhancers and analysis of regulatory mechanisms can be performed using CRISPR/Cas9 and its derivative technologies | [110, 125] |
| Oligonucleotide method | LNAs | Targeting eRNA to inhibit enhancer activity | [127] |

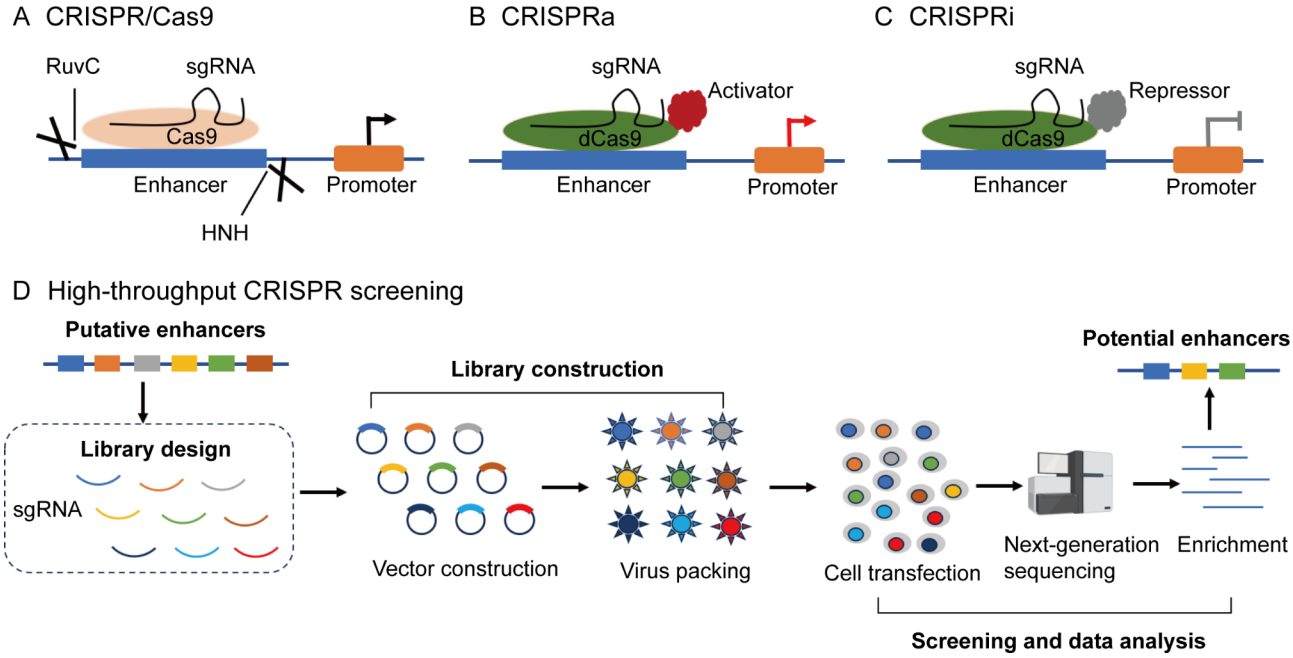


**Fig. 3** Enhancer screening and functional annotation using CRISPR/Cas technology. **A** CRISPR/Cas9 technology is performed to delete an enhancer region under the guidance of sgRNA. **B** CRISPRa for enhancer activation to promote transcription. **C** CRISPRi for enhancer interference to repress transcription. Both CRISPRa and CRISPRi use dCas9 fused to an effector protein (activator or repressor). **D** High-throughput screening of enhancers, including sgRNA design to generate sgRNA libraries for screening, sgRNA delivery via lentiviral vectors and stable integration into target cells, next-generation sequencing analysis and screening

VP64 depends on the sustained recruitment of cofactors, such as the histone acetyltransferase p300, dCas9-VP64 can be used to activate enhancer activity [114]. Hilton et al. developed the dCas9-p300 system, which can achieve instant and efficient acetylation of histones when targeting enhancers, thereby achieving transcriptional activation of target genes. In addition, the dCas9-p300 system can achieve high specificity in transcriptional activation of target genes and has a higher transcriptional activation ability than dCas9-VP64 [115]. These results demonstrate that dCas9-p300 is a robust tool for modulating histone acetylation and activating enhancer activity. To augment transcriptional activation, the dCas9-SAM system has been engineered, building upon the dCas9-VP64 platform. This system is capable of modifying sgRNAs and fusing multiple transcriptional activation domains to enhance the recruitment of the RNA polymerase complex [116–118]. This improvement not only enhances the activation effect, but also makes it possible to activate multiple genes at the same time.

CRISPRi was initially found to block RNA polymerase elongation by targeted the binding between the dCas9-sgRNA complex and target DNA, thereby inhibiting the transcription of target genes [119]. This approach disrupts gene transcription by physically interfering with the binding of TFs to enhancers [120]. Nevertheless, the effectiveness of this dCas9-based approach in achieving gene repression in mammalian cells is suboptimal. To increase the inhibition efficiency, Gilbert et al. fused dCas9 with several repressive effectors and found that the dCas9-KRAB fusion had higher repression efficiency [121]. Using dCas9-KRAB to target enhancer activity in human T cells, enhancers were screened out to regulate the expression of *TNF* and *LTA* [122]. dCas9-LSD1, as a complementary gene suppression system to the dCas9-p300 activation system, has a more stable enhancer suppression effect and higher targeting specificity than dCas9-KRAB. Studies have shown that dCas9-LSD1 induces histone demethylation of enhancers and leads to a significant decrease in H3K4me2 and H3K27ac markers [123, 124].

### *CRISPR screens for enhancers*

CRISPR screening, as one of its applications, involves genome-wide sgRNA library construction, functional screening and enrichment, PCR amplification, and deep sequencing analysis to discover and screen candidate genes [116]. In the study of enhancers, hundreds of sgRNAs are arranged within putative enhancer regions to disrupt the function of enhancers, and then the functional sequence of the enhancer and the transcriptional binding site are evaluated by the abundance of sgRNAs [125]. In addition, sgRNAs targeting different enhancers are delivered to a pool of cells and the activity of the enhancers is assessed by changes in putative target gene expression [72]. CRISPR screening provides a

high-throughput and efficient tool for enhancer screening (Fig. 3D). However, due to the large number of enhancers in the genome, high-throughput CRISPR screens integrating multi-omics methods to identify candidate enhancers remain challenging.

#### *Locked nucleic acids (LNAs)*

LNAs are special antisense oligonucleotides modified with a phosphorothioate backbone, and their 2′ C and 4′ C atoms are linked by a methylene bridge, which increases the affinity of LNA to complementary sequences. LNAs also have higher sequence specificity, biological activity, and thermal stability [126]. These properties enable LNAs as inhibitors of non-coding RNAs (ncRNAs). Since eRNAs are a type of non-coding RNAs transcribed from enhancers, the use of LNAs to target eRNAs to inhibit enhancer activity has recently been shown to be effective [127]. Zhang et al. designed three LNAs GapmeR to knockdown *NET1*-associated eRNA (*NET1e*) and further verified the function of *NET1e* in breast cancer cell proliferation [127]. However, the inhibitory effect of LNAs is short-lived, and it is unclear to what extent LNAs can interfere with enhancer activity.

## Enhancers in disease

Enhancers are recognized as pivotal regulators that govern the precision and dynamic equilibrium of gene transcription, and enhancer abnormalities directly affect stability, expression intensity, and specificity on genes [10]. Genomic imprinting, a complex epigenetic phenomenon involving in the precise regulation of imprinted gene expression, is important for growth, development, and disease [128]. Recent studies have shown that enhancers interact with differentially methylated regions (DMRs) of imprinted genes to influence methylation status, thereby regulating imprinted gene expression patterns [129, 130]. In addition, enhancer dysfunction has been implicated in the pathogenesis of many common human diseases, such as cancer and osteoporosis [131, 132]. Therefore, the study of the relationship between enhancer dysfunction and disease is one of the core topics in modern biology, and in-depth research into the regulatory mechanisms of enhancers will provide a new approach to disease prevention and treatment.

### Multiple patterns of aberrant enhancer

The aberrations in enhancer are mainly caused by point mutations, structural variations (copy number variations or chromosomal rearrangements), and epigenetic changes (enhancer DNA methylation or histone modifications).

#### *Point mutations*

Point mutations in enhancer sequences can alter transcription binding sites, resulting in abnormal enhancer-transcription factor binding and dysregulation of gene transcription. In cancer, point mutations in enhancers may affect the binding sites with transcription factors or tumor suppressor genes, leading to inappropriate expression of tumor suppressor genes. Another possibility is that point mutations within enhancers may increase the binding sites with transcription factor, thus promoting oncogene expression (Fig. 4A) [12, 133]. In addition to cancer, enhancer point mutations can lead to the development of congenital disorders, including intellectual disability [134], Pierre Robin syndrome [135], congenital heart disease [136], and neurological disorders [137]. A recent study found that mutations in patients with intellectual disability were significantly enriched in fetal brain-specific enhancers that influence neurogenesis [134].

#### *Structural variations*

Under normal states, enhancers specifically regulate the expression of target genes, but structural variations in enhancers can lead to mistargeting, resulting in non-specific regulation by enhancers [138]. The structural variation of enhancers caused by chromosomal rearrangement can lead to "enhancer hijacking" events [139]. Enhancer hijacking refers to the transfer of enhancers on the same or different chromosomes to the vicinity of oncogenes, resulting in the juxtaposition of ectopic enhancers and proto-oncogenes, thereby driving oncogene activation (Fig. 4B left) [140]. Recently, whole-genome sequencing analysis of more than 1200 cancer genomes has revealed that hundreds of genes exhibit expression changes within 100 kb of structural variation breakpoints, some of which may be due to enhancer hijacking [141]. Enhancer hijacking is an important mechanism driving oncogene activation, which results in the aberrant expression of oncogenes. In addition, copy number variations can affect TADs, and disruption of TAD boundaries can also cause enhancers to ectopically activate genes, leading to the onset of some rare diseases (Fig. 4B right) [142–144].

#### *Enhancer methylation/demethylation*

DNA methylation is a common repressive epigenetic modification in the genome that regulates gene expression and alters genetic manifestations without altering the genomic DNA sequence. Methylation modification of enhancer regions can alter enhancer activity (Fig. 4C). In a study based on a large-scale analysis of 33 types of cancer in the TCGA, a total of 12,559 active enhancers were identified. The study further identified enhancer methylation regions and compared differentially methylated enhancers between cancer and normal tissues, and

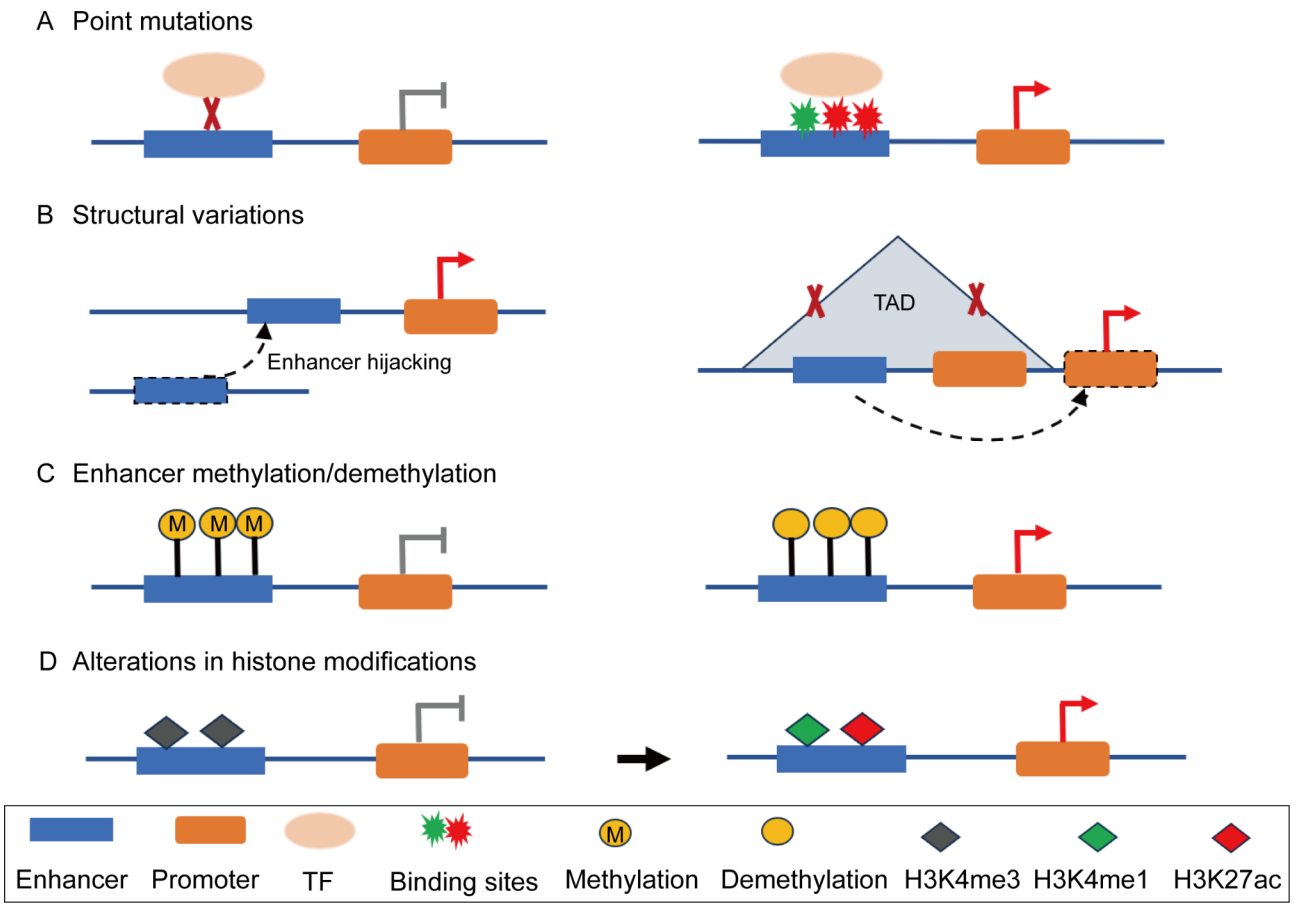


**Fig. 4** Multiple patterns of enhancer aberrant. **A** Point mutations, including loss of intrinsic binding sites (left) and addition of other binding sites (right). **B** Structural variations. "Enhancer hijacking" caused by chromosomal rearrangement (left). Copy number variations affecting TADs (right). **C** Enhancer methylation (left) and demethylation (right). **D** Alterations in histone modifications can alter the status of enhancers, thereby affecting transcription

found that in most cancer types, the degree of enhancer methylation is inversely correlated with enhancer activity [145]. Enhancer hypomethylation can promote abnormal activation of enhancers and promote cancer progression [146].

#### *Alterations in histone modifications*

Histone modification is an important epigenetic regulatory mechanism that can regulate gene expression by affecting chromatin compaction, DNA accessibility, and transcription factor binding capacity. Histone modifications include phosphorylation, acetylation, ubiquitination, and most commonly methylation or demethylation. Histone modifications, such as H3K4me1 and H3K27ac, can be used to localize, label, and characterize enhancer status [147]. Alterations in histone modifications can alter enhancer activity and promote aberrant gain of enhancer function, leading to abnormal activation of signaling pathways and abnormal gene expression, thereby affecting the onset or progression of disease (Fig. 4D) [148]. Histone modification H3K4me1 is mainly catalyzed by a complex with MLL3 or MLL4 as the core in the lysine methyltransferase 2 (KMT2) protein family [149]. Studies have shown that MLL3 or MLL4 mutations alter H3K4me1 modification, leading to aberrant enhancer activity, which is a potential mechanism for tumor cell invasion and metastasis [148, 150, 151].

### Prospects of enhancers in clinical application

Human diseases caused by enhancer dysfunction are often called enhanceropathies [152]. Given the widespread occurrence and intricate nature of enhancer-opathies, comprehensive research into enhancers holds significant potential for advancing disease diagnostics and shaping therapeutic strategies.

#### *Diagnostic biomarkers*

Genome-wide association studies (GWAS) have revealed that 93% of SNPs associated with complex diseases are fall in non-coding regulatory regions, with 64% of the sites within enhancer regions [153]. Using targeted capture and multiplex sequencing of the target enhancer regions is helpful to quickly find tumor-related mutation sites and to obtain new diagnostic markers. However, the

functional characterization of SNPs identified by GWAS remains an open challenge.

#### *The development of targeted medications*

Due to the strong correlation between diseases and enhancers, drug development and treatment strategies targeting enhancers have received increasing attention. The BET protein family is an important family of transcriptional activator proteins, most of which are located in the enhancer region. Some important oncogenes such as MYC, BCL2 and CDK6 are regulated by enhancers that bind to the BET proteins [154, 155]. BRD4 is a member of the BET family of proteins, which are enriched in active enhancer regions in various cancers and directly regulate MYC protein expression by binding to the transcription factor MED1. The small molecule inhibitor JQ1, which targets BRD4, can inhibit enhancer activity and reduce the level of binding between enhancers and MED1. The BET-bromodomain inhibitor JQ1 can disrupt enhancer function and has good therapeutic prospects. Studies have shown that treating leukemia cells with JQ1 inhibitors can reduce the expression of proto-oncogenes, such as *MYC* and *BCL2* [156]. In the mouse model of atherosclerosis, JQ1 treatment can reduce the occurrence of early atherosclerosis [157]. Other BET inhibitors, such as i-BET151 and OTX015, have also shown good therapeutic effects in clinical trials [158–160]. Although the exploration of BET-bromodomain inhibitors as therapeutic drugs is unanimously optimistic, some existing problems need to be considered and resolved, such as the timing of JQ1 treatment. Studies have found that mice treated with JQ1 in the early stages of acute kidney injury in mice had higher mortality, while mice treated later had less fibrosis [161]. The exploration on the related enhancers may help to optimize the medication schedules for BET inhibitors.

#### *Gene therapy*

The efficacy and safety of gene therapy strategies depend primarily on the precise temporal and spatial expression of key disease genes. Enhancers play a pivotal role in the precise regulation of gene expression, and targeted gene therapies that modulate enhancer function have achieved significant progress in the treatment of genetic disorders. CRISPR/Cas9 gene editing technology can target and precisely modify enhancer mutation sites to achieve gene therapy effects. In recent years, a breakthrough in the treatment of patients with severe thalassemia has been achieved using CRISPR/Cas9 gene editing technology by targeting enhancers [162, 163]. Targeting enhancers offers new ideas for gene therapy and will provide a safer and more effective treatment strategy for patients with diseases. However, how to ensure precise regulation of enhancers in disease deserves further exploration.

## Conclusions and future perspectives

Over the past few decades, research on non-coding regions, especially enhancer regions, has received a great deal of attention and it has become clear that the role of enhancers in development and disease cannot be ignored. Enhancers hold great promise for understanding disease pathogenesis and identifying genetic biomarkers or potential therapeutic targets. This work systematically summarizes the biological characteristics of enhancers, the current technical methods of enhancer research, and the application value of enhancers in disease treatment.

Currently, the continued advancement of high-throughput sequencing technology has greatly improved our ability to identify and study enhancers, and large-scale enhancers have been screened and identified. Despite this tremendous progress, there are still many problems to be solved in studying how enhancers work. For example, (1) How to accurately identify functional enhancers? Enhancers are often defined in indirect ways, such as chromatin accessibility, eRNA transcript levels, or histone modifications associated with enhancer activity. MPRA allows large-scale testing of sequences with potential enhancer activity in the genome [92]. The Encyclopedia of DNA Elements (ENCODE) project provides a wealth of sequencing data for human and mouse cell lines and tissues, including ChIP-seq data of histones and TFs, ATAC-seq data, Hi-C data, etc [164]. The Roadmap Epigenomics project contains thousands of genome-wide epigenomic datasets, describing the epigenomes of a variety of different human tissue and cell types [165]. The Functional Annotation of the Mammalian Genome 5 (FANTOM5) project provides annotations of enhancers in the human and mouse genomes. Currently, about 65,359 human enhancers and 44,000 mouse enhancers have been annotated by FANTOM5 [7]. The integration of these datasets facilitates the annotation of enhancers within specific tissues or cell types. Improving the precision of enhancer annotation remains an important challenge that requires further investigation and development of more sophisticated methodologies. (2) How to elucidate the interactions between enhancers and other regulatory elements? Gene expression regulation requires the cooperation of multiple regulatory elements or even multiple enhancers working together to increase the transcription level of a gene [166, 167]. Understanding the interactions between enhancers and other regulatory elements and unraveling the complex regulatory networks between enhancers and genes is still a challenge. (3) Search for target genes regulated by enhancers. One enhancer can regulate more than one target gene, and target genes can be regulated by different enhancers [7]. Accurately identifying target genes regulated by enhancers poses a significant challenge in the field of genomics, necessitating the development of more refined screening

techniques. A recent study based on the large amount of data generated by transcriptomics and genomics, combined with deep learning models, has greatly improved the prediction accuracy on enhancer target genes [168]. (4) To understand the role of TFs in enhancer regulation. TFs are involved in enhancer-regulated transcription in both normal and pathological conditions. To fully understand the molecular mechanism of enhancer regulation, it is necessary to study the synergistic effects of enhancers and TFs. Current research only focuses on a small number of TFs, and more TFs deserve more attention and analysis [169, 170].

The comprehensive and systemic role of enhancers in regulating gene expression across the genome is not yet fully understood. Compared to the large number of enhancers in the human genome (~65,359 annotated by FANTOM) [7], the existing research on enhancer function represents only a fraction of the vast and complex landscape of their regulatory roles within the genome. Extensive functional studies of individual enhancers will be important in future work, especially given the strong correlation between enhancers and diseases. As our understanding of the role of enhancers in disease expands, a crucial future objective is to delineate the specific proteins that interact with functional enhancers, identify their potential regulatory motifs, and pinpoint specific therapeutic targets [171, 172]. Progress in these directions will facilitate the systematic dissection of enhancer regulation and function by using the methods described above, including high-throughput CRISPR screens, MPRAS, and gene editing tools. Our current comprehension of enhancers, combined with the sophisticated application of the aforementioned enhancer research methodologies, holds the potential to unravel the complex mechanisms underlying the enhancer-mediated transcriptional regulation. Overall, we endeavor to make significant strides in demystifying the complex interplay of enhancer transcription and function, thereby establishing clearer connections between enhancers, gene regulation, development, and disease.

**Abbreviations**

| | |
|---|---|
| ATAC-seq | Assay for transposase-accessible chromatin with high throughput sequencing |
| CAGE | Cap analysis of gene expression |
| ChIA-PET | Chromatin interaction analysis by paired-end tag sequencing |
| ChIP-seq | Chromatin immunoprecipitation followed by sequencing |
| CUT&RUN | Cleavage under targets and release using nuclease |
| CUT&Tag | Cleavage under targets and tagmentation |
| DNase I | Sensitivity to deoxyribonuclease I |
| E-P | Enhancer-promoter |
| EPI | Enhancer-promoter interactions |
| eRNAs | Enhancer RNAs |
| FAIRE-seq | Formaldehyde-assisted isolation of regulatory elements sequencing |
| GRO-seq | Global run-on sequencing |
| H3K27ac | Acetylation of histone H3 lysine 27 |
| H3K4me1 | Monomethylation of histone H3 lysine 4 |
| H3K4me3 | Trimethylation of histone H3 lysine 4 |
| Hi-C | High-throughput chromosome conformation capture |
| HiChIP | In situ Hi-C followed by chromatin immunoprecipitation |
| HiCuT | Hi-C coupled chromatin cleavage and tagmentation |
| LNAs | Locked nucleic acids |
| MNase-seq | Micrococcal nuclease sequencing |
| MPRAs | Massively parallel reporter assays |
| NDR | Nucleosome-depleted region |
| Pol II | RNA polymerase II |
| RNA-seq | RNA sequencing |
| sgRNA | Single guide RNA |
| SNPs | Single nucleotide polymorphisms |
| STARR-seq | Self-transcribing active regulatory region sequencing |
| TADs | Topologically associated domains |
| TF | Transcription factor |

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**Data availability**
No datasets were generated or analysed during the current study.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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