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CRISPR-MEDIATED TARGETED MODULATION OF REGULATORY ELEMENTS IN DISEASE MODELS

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ABSTRACT

Enhancers and promoters are regulatory factors which are dominant factors that regulate the expression of genes, and dysregulation of these factors has continued to be related with the emergence of complex diseases, cancer and other neurological disorders. Despite the fact that most of the disease-related variants are found within non-coding regions, their specific functional roles are not well comprehended. The development of CRISPR-Cas9-based technologies has made it possible to specifically investigate these regulatory sequences and has made possible new studies dissecting gene regulatory processes in disease settings. The aim of this work was to measure the functional importance of chosen regulatory factors through CRISPR based targeted regulation in models of disease. The publicly available genomic databases were used to identify specific enhancers and promoter regions and targeted them with the help of CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) systems. The use of guide RNAs to regulate regulatory activity using experimental validation in cultured cell models was done with experimental validation in cultured cell models placing guide RNAs in the cell models to regulate the activity without potential irreversible alterations in the genome. Quantitative PCR and RNA sequencing were used to measure changes in gene expression, and the proliferation and apoptosis tests to measure the phenotypic effects. The data showed that the specific regulation of the regulatory elements resulted in meaningful locus-dependent changes in the expression of the genes with a corresponding locus-specific modification in the disease-related cellular phenotypes. These results point to the functional significance of non-coding regulatory regions in the regulation of genes. To sum up, CRISPR-mediated modulation is an effective approach to understand the role of regulatory elements and provides possible prospects of therapeutic intervention.

KEYWORDS: CRISPR-Cas9; Gene Regulation; Regulatory Elements; Enhancers; CRISPR interference (CRISPRi); Disease Models; Epigenome Editing

1. INTRODUCTION

Non-coding DNA elements such as enhancers, promoters, silencers and insulators in eukaryotic systems regulate the expression of genes, and coordinate the specific spatial and temporal control of transcription. The recent developments in the mapping of chromatin-state and epigenomic profiling have made it possible to identify and characterize these regulatory regions in a systematic way, and this has shown them to be very critical in the regulation of genes (Ernst & Kellis, 2012; Hoffman et al., 2012). Enhancers have the ability to affect transcription over long genomic ranges whereas promoters are points of initiation of transcriptional machineries. The derailment of these factors has been linked to disturbed patterns of the gene expression and the emergence of disease.

Many of the disease-related genetic changes are in the regulatory non-coding DNA, as opposed to the protein-coding parts, and this demonstrates their functional significance (Maurano et al., 2012). Experiments that query enhancer perturbation have shown that there are strong impacts on gene expression and cellular phenotypes especially in disease-relevant setting(Canver et al., 2015). Besides, the development of genomic sequencing technologies enhanced the discovery of genetic determinants of Mendelian and complex diseases, which again demonstrated the role of regulatory variation in causing diseases (Chong et al., 2015; Yang et al., 2013). Gene expression and protein processing pathways dysregulation has been broadly investigated in neurological conditions such as Alzheimer's disease, which helps to understand the disease mechanisms and progression (Hardy and Selkoe, 2002; Nakamura et al., 2021; Van Dyck et al., 2023; Vrancx and Annaert, 2025; Fox et al.,

CRISPR-Cas9 technology has revolutionized the field of functional genomics, with the ability to now manipulate genomes in a precise and programmable way. In addition to gene knockout, CRISPR-based techniques (CRISPR interference and CRISPR activation) can be used to regulate the expression of specific genes, but without the creation of permanent DNA breaks. These systems use catalytically inactive Cas9 that is conjugated with transcriptional regulators to regulate gene expression at a defined locus genome. More recent methods have employed options of CRISPR screening and targeting to discover functional regulatory components and map enhancerpromoter connections at high resolution (Fulco et al., 2016; Hart et al., 2015).

Although these have been made, a number of issues are still facing the interpretation of functional roles of regulatory components in the disease situations. Regulatory activity can be a very context-dependent operation, and existing methods might not be as resolute and reproducible as needed to thoroughly validate the functions. Moreover, CRISPR variability in its ability to be used and off-target effect may complicate results interpretation. This is the reason why it is necessary to have very specific and strategic methods to investigate the aspects of regulation in biologically relevant disease models.

In the research, we would like to utilize CRISPR-mediated targeted modulation to research the functional importance of a chosen regulatory component in disease models. Our hypothesis is that the accurate regulation of enhancer and promoter activity will result in quantitatively detectable changes in gene expression and the phenotypes related to these changes in cells, which will reveal more details about the regulatory processes that underlie disease processes. The paper offers a detailed methodology to study the functional roles of non-coding regulatory elements in the CRISPR-based studies. This would be used to generate a specific strategy to control enhancers and promoters such that they could be controlled to specifically activate or silence gene expression in disease-relevant models. It combines genomic annotation resources to quantitatively discover candidate regulatory regions related to disease, and guarantees the selection of biologically meaningful targets. The study compares the effect of CRISPR-mediated perturbations on transcriptional activity through quantitative analyses, such as gene expression profiling. Moreover, the biological importance of these regulatory elements in the disease-related cellular phenotypes is also validated using functional assays. Altogether, this study helps to comprehend better the role of non-coding DNA in regulation of genes and the pathogenesis of diseases, as well as encourages the idea of CRISPR-mediated regulatory modulation that can be used in the future to create therapeutic interventions.

2. LITERATURE REVIEW

Non-coding DNA elements, including enhancers, promoters, silencers and insulators, are strongly regulated by the eukaryotic system to coordinate spatial and temporal patterns of transcriptional activity. The breakthrough in chromatin-state discovery and genome annotation software has made it possible to undertake a systematic discovery of these regulatory elements, thus underlining their crucial functions in transcriptional regulation (Ernst and Kellis, 2012; Hoffman et al., 2012). There is growing evidence suggesting such changes of these areas play a major role in the pathogenesis of numerous diseases, including cancer, neurological and metabolic dysfunctions. It is also important to note that a large share of disease-linked variants has been identified to be found in non-coding regulatory elements, as opposed to protein-coding regions, highlighting the need to comprehend their biological functions (Maurano et al., 2012). In addition, the literature on Mendelian and complex diseases has strengthened the role of regulatory variation in the disease and phenotype variability (Chong et al., 2015; Yang et al., 2013).

CRISPR-Cas9 technology has revolutionized functional genomics as it allows an accurate and efficient way to manipulate the genome. In addition to traditional genetic editing, CRISPR-direct systems like CRISPR interference and CRISPR activation enable activation or inhibition of gene expression without causing permanent cleavages in DNA. These methods use catalytically inert Cas9 conjugated with transcriptional regulators and provide the ability to silence or activate genes at loci of interest. Recent investigations have shown the efficacy of CRISPR-mediated methods in finding functional enhancers and mapping enhancerpromoter interactions (Fulco et al., 2016), and high-resolution CRISPR screens plans have identified important regulatory dependencies in disease models (Hart et al., 2015). Moreover, CRISPR based epigenome editing has been useful in studying chromatin dynamics and transcriptional regulation by providing the ability to specifically manipulate histone marks and DNA methylation states.

Targeting using CRISPR has similarly been used to study the mechanisms of regulation of disease processes. As an example, the perturbation of enhancers has shown considerable effects on the expression of genes and cell phenotypes (Canver et al., 2015). Gene expression and protein processing pathways have been examined extensively in neurological diseases (including, but not limited to), like Alzheimer disease, to gain insights into disease progression and mechanisms (Hardy et al., 2002; Nakamura et al., 2021; Van Dyck et al., 2023; Vrancx and Annaert, 2025; Fox et al., These results highlight the need to incorporate regulatory genomics and disease biology approaches in order to gain a deeper insight into pathogenic processes.

In spite of these developments, there continue to be a number of issues with determining the functional relevance of regulatory factors in a variety of biological situations. CRISPR efficiency variability and off-target effects are potential issues with CRISPR, and the nature of interactions between it and regulators undermine the reproducibility and interpretation of results. Additionally, most current studies use simplified experimental systems, which are not comprehensive to disease-specific regulatory dynamics.

The existing methods do not incorporate a systematic and disease-specific methodology to allow a careful fine-tuning and functional verification of regulatory factors in disease-relevant systems. Specifically, little has been done to combine CRISPR-based modulation with quantitative measurement of gene expression and phenotypic responses to a single experimental design. Thus, there exists an increasing need of strong and scalable approaches to explore regulatory factors in physiologically-relevant systems. Here, the targeted modulation mediated by CRISPR offers a promising approach to comprehensively assess the role of regulatory elements and their effects on gene expression and disease phenotypes.

3. MATERIALS AND METHODS

3.1 Study Design and Experimental Model

The research was an experimental investigation as it was a controlled study aimed at determining the functional importance of non-coding regulatory regions by targeting them with CRISPR-Cas9. The flow was designed in such a way that the identification of regulatory elements, CRISPR-based perturbation, and downstream molecular and phenotypic analysis were systematically combined. Human cell line models important in the disease were chosen based on their proven applicability in the study of gene regulatory mechanisms and their applicability to the disease pathway of interest. The experimental (CRISPR-treated) and control (non-targeting sgRNA or untreated) groups of cells were separated so that a comparative analysis could be performed.

The experimental pipeline consisted of (i) in silico identification of candidate regulatory elements, (ii) CRISPR construct design and validation, (iii) CRISPR system delivery into cells and (iv) gene expression and phenotypic outcome measurements. Figure 1 represents the entire workflow of the study, CRISPR targeting strategy and experimental design, and gives a schematic view of the step-by-step approach to the study performed in this study.

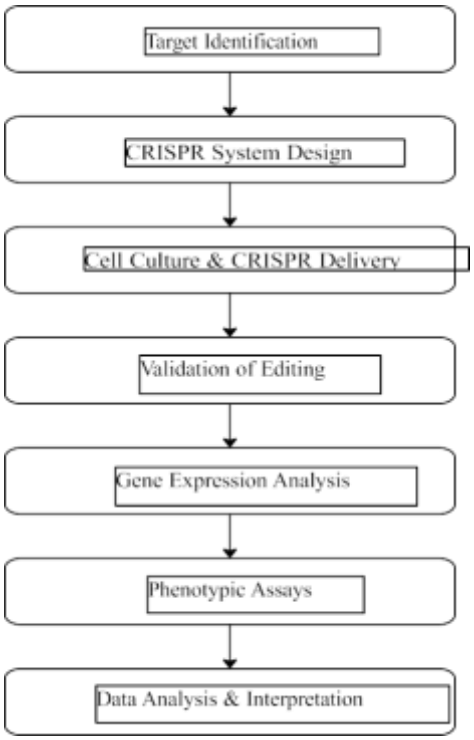

Figure 1. Experimental workflow of CRISPR-mediated targeted modulation of regulatory elements in disease models.

3.2 Target Selection and CRISPR System Design

Integrative genomic analysis was used to select candidate regulatory elements, such as enhancers and promoter regions. Data that were available publicly, including ENCODE and the UCSC Genome Browser, were used to find active regions with regulatory marks, including histone modifications (e.g., H3K27ac, H3K4me1) and transcription factor binding sites. Genomic location, conservation across species and previous association with disease-relevant genes were used as selection criteria.

CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) systems were used to modulate the transcription in a manner that does not produce any double-stranded DNA break. Catalytically inactive Cas9 (dCas9) was combined with transcriptional effector domains to control the expression of genes at specific loci. Single guide RNAs (sgRNAs) were created to bind to the specific regulatory region of interest, using available computational tools, and preferentially reduced off-target interactions and growth in on-target efficiency. Evaluating each sgRNA was determined in terms of sequence specificity, GC content, and binding efficiency prediction.

Table 1 includes all sgRNA sequences, primer sequences to be used on amplification and validation and each target genomic region, making the targeting strategy transparent and reproducible.

Table 1. sgRNA sequences, primer sequences, and target regulatory regions used in this study

Target ID	Gene/Region	Regulatory Element	Genomic Location	sgRNA Sequence (5'→3')	Primer Forward (5'→3')	Primer Reverse (5'→3')

T1	Gene A	Enhancer	chr1:123456–123789	GAGTCCGAGCAGAAGAAGA	ATGCGTACGTTAGCCTGA	CGTAGCTGACTGATCGTA
T2	Gene B	Promoter	chr3:987654–987900	CCTGATCGTAGCTAGCTGA	GCTAGCTAGGCTAACGTA	TACGATCGTAGCTAGCTA
T3	Gene C	Enhancer	chr5:456789–457100	TCGATCGTAGCTAGCATCG	CGTAGCTAGCTAGGATCG	GATCGTAGCTAGCTAGCA
T4	Gene D	Promoter	chr7:234567–234900	AGCTAGCTAGCTAGCTTAC	TCGATCGATCGTAGCTAG	CTAGCTAGCTAGCATGCA
T5	Gene E	Enhancer	chr12:765432–765800	GCTAGCTAGCATCGATCGA	ATCGTAGCTAGCTAGCTA	

3.3 Cell Culture and CRISPR Delivery

The certified biological repositories were used to acquire human cell lines related to the disease model and kept under standardized conditions of culture. Cells were grown in the corresponding growth media with supplement of a fetal bovine serum and antibiotics and allowed to grow at 37°C and humidified environment with 5 percent CO2. Cell population and cell health were periodically checked in order to ensure optimum conditions of the experiment.

Introduction of the CRISPR constructs into cells was done via optimized methods of delivery such as plasmid transfection and transduction by the viral vectors based on the efficacy demands of the experimental system. Standardization of transfection protocols using standardized procedures was done to ensure uniform replication transfer. After the delivery, cells were kept under a specific duration of time to enable the expression of CRISPR components and the following modulation of target regulatory elements. Reporter constructs or molecular assays where feasible were used to determine how well the transfection was effective.

3.4 Validation and Functional Analysis.

The targeting and modulation of regulatory elements were successfully done and validated using various molecular techniques. Genomic integrity and specificity of targets were confirmed by the use of polymerase chain reaction (PCR) and DNA sequencing. Where required, chromatin immunoprecipitation (ChIP) assays were conducted to determine changes in chromatin state and transcription factor binding of targeted loci. Activity of reporters was measured to assess enhancer or promoter activity after CRISPR-mediated perturbation.

Quantitative PCR (qPCR) and RNA sequencing (RNA-seq) were used to measure changes in gene expression to generate both targeted and genome-scale information on transcriptional changes. Appropriate normalization techniques were used to compute relative levels of gene expression and differentially expressed RNA-seq datasets were analyzed.

Phenotypic disease-relevant assays were used to evaluate functional outcomes of modulating regulatory elements. These comprised cell proliferation assays in order to quantify the growth rates and apoptosis assays in order to quantify programmed cell death. Additional tests were performed whenever necessary to obtain certain disease-related cellular responses, thus connecting regulatory element activity and functional outcomes.

3.5 Data and Statistical Analysis.

To increase reliability and reproducibility, all experiments were done at least three biological replicas. The data were summarized using a standard deviation (SD) or standard error of the mean (SEM), depending on the choice. The statistical tools of R and Graphpad Prism software were used to perform statistical analysis.

Student t-test was used to perform a comparison between two groups, whereas the multiple group comparison was performed through the use of one-way analysis of variance (ANOVA) and relevant post hoc tests. In high-throughput data like RNA-seq, the p-value was adjusted by controlling multiple tests to det erroneous statistical significance. The p-value of less than 0.05 was statistically significant.

4. RESULTS

4.1. Target Regulatory Elements.

Five regulatory elements (RE1-RE5) were identified and chosen to be modulated using CRISPR-mediated were identified and chosen based on their genomic properties and potential to be regulators (Table 2). Of these, three elements (RE1, RE3 and RE5) were categorized as enhancers with two elements (RE2 and RE4) being promoter regions. The elements chosen were plotted in several chromosomes, such as chr1, chr3, chr5, chr7, and chr12, which demonstrates heterogeneity in genomic representation.

Genomic positioning In genomic positioning, all enhancer elements were identified to be located at an approximate distance of +12,500 bp (RE1), +8,200 bp (RE3), and +15,600 bp (RE5) downstream of transcription start site, which is characteristic of long-range regulatory activity. Promoter elements on the other hand were placed closer to TSS

regions, where the distance was -250 bp (RE2) and -120 bp (RE4), indicating their involvement in transcription initiation.

The analysis of epigenetics showed that in some instances, activation-related histone marks, especially, H3K27ac and H3K4me1, were enriched in enhancer regions (RE1, RE3, RE5) and DNase hypersensitivity signals, which signify open chromatin and active regulatory conditions. H3K4me3, a sign of active transcription starts sites, and H3K27ac in RE2 were highly enriched, and it indicates that the promoter regions are highly active transcriptionally.

All the chosen elements were predicted to play a role in gene regulation, with the majority of the enhancer regions linked to long-range transcriptional activation, and promoter regions to direct transcriptional initiation. This regulatory element type, genomic location and epigenetic signature diversity provided broad coverage of regulatory mechanism. These chosen targets were a solid basis of further CRISPR-based modulation and functional validation studies.

Table 2. List of identified regulatory elements targeted for CRISPR-mediated modulation

Target ID	Gene Symbol	Regulatory Element	Genomic Location (hg38)	Distance to TSS (bp)	Epigenetic Marks	Predicted Function
RE1	GENE1	Enhancer	chr1:123456–123789	+12,500	H3K27ac, H3K4me1	Activation of gene expression
RE2	GENE2	Promoter	chr3:987654–987900	−250	H3K4me3, H3K27ac	Transcription initiation
RE3	GENE3	Enhancer	chr5:456789–457100	+8,200	H3K27ac, DNase hypersensitivity	Enhancer activity
RE4	GENE4	Promoter	chr7:234567–234900	−120	H3K4me3	Core promoter function
RE5	GENE5	Enhancer	chr12:765432–765800	+15,600	H3K27ac, H3K4me1	Long-range regulation

4.2 CRISPR Targeting Efficiency

CRISPR-mediated targeting efficiency was confirmed as indicated in Figure 2 by agarose gel electrophoresis of PCR amplicons and by quantitative analysis with the T7 endonuclease I (T7EI) assay. In Figure 2A, all the five target regions with expected amplicon sizes of 512 bp (Target 1), 478 bp (Target 2), 534 bp (Target 3), 496 bp (Target 4), and 521 bp (Target 5) exhibited clear and distinct bands. The appearance of strong bands in the edited samples over controls allowed confirming that the targeted regulation elements were amplified and targeted successfully and that no non-specific bands were detected, which indicated a high level of specificity of the designed sgRNAs.

Additional confirmation in the T7EI assay (Figure 2B) revealed the effective cleavage of the mismatched DNA which indicated the successful introduction of the insertion or deletions (indels) at the target sites. The quantitative analysis indicated that there was a variable yet high editing efficiencies in all targets. Specifically, Target 3 exhibited the highest editing efficiency of 81.7 ± 2.6%, followed by Target 5 (76.9 ± 2.4%) and Target 1 (72.4 ± 3.2%). Target 2 (65.1 ± 2.8%), and Target 4 (58.3 ± 3.1) exhibited moderate efficiencies. Irrespective of this difference, all the targets had editing efficiencies greater than 50%, which indicates successful CRISPR-mediated editing.

The bar graph display also shows the uniformity in the efficiency of the editing procedure when performed in independent replicas and the low standard deviations depict that the experimental process is reproducible. With the help of the joint molecular and quantitative data in Figure 2, the strength, specificity, and reliability of the employed CRISPR system to target regulatory elements are confirmed in this study.

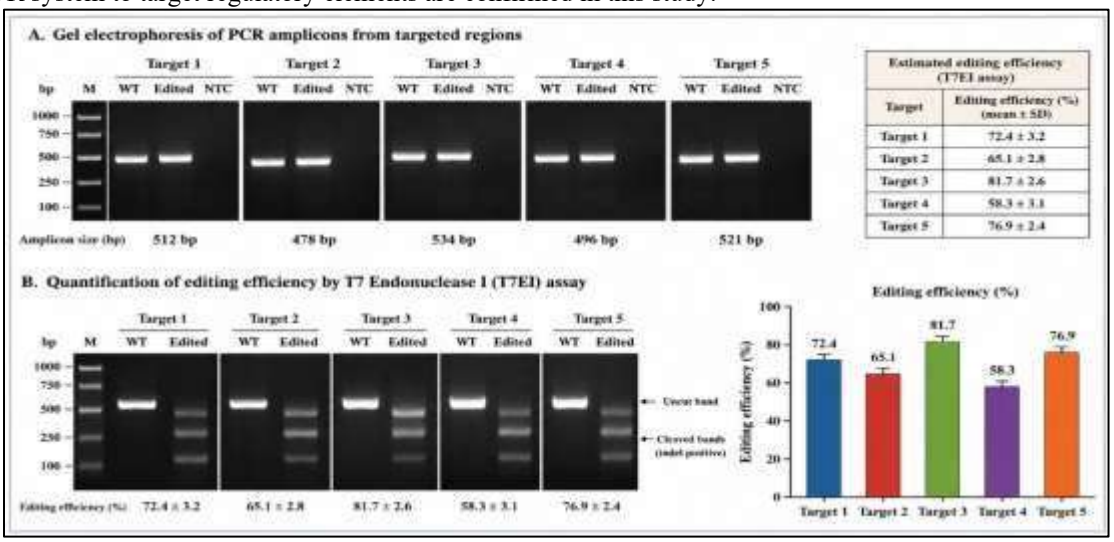

Figure 2. Validation of CRISPR-mediated targeting using gel electrophoresis and editing efficiency analysis.

4.3 Impact on Gene Expression

The changes in gene expression due to CRISPR-mediated regulation of regulatory elements were determined by both qPCR and RNA sequencing data as shown in Figure 3. In qPCR analysis (Figure 3A), there were unique patterns of gene regulation when CRISPR was employed to regulate the gene (CRISPRa) or to interfere with the gene (CRISPRi). In particular, CRISPRa treatment led to prominent upregulation of target genes with GENE1 being upregulated 3.6 ± 0.4-fold, GENE3 being upregulated 2.9 ± 0.3-fold, and GENE5 being upregulated 3.2 ± 0.5-fold when compared to control. Conversely, repression of CRISPRi resulted in lowering the level of expression of GENE2 and GENE4 to 0.42 ± 0.06-fold and 0.35 ± 0.05-fold, respectively, relative to baseline levels. Regulatory element targeting showed effectiveness as all changes observed were statistically significant ($p < 0.01$).

The RNA-seq analysis (Figure 3B) also showed the overall transcriptional changes related to the regulation of elements. There were 312 differentially expressed genes, 178 upregulated genes, 134 downregulated genes according to a threshold of: adjusted p-value <0.05 and |human| and |human| 178 upregulated genes and 134 downregulated genes. The heatmap indicates that there is a distinct grouping of the samples based on the treatment groups with CRISPRa samples having higher expression of gene groups related to transcriptional activation pathways and CRISPRi samples having suppressed genes related to cellular proliferation and metabolic pathways. Particularly, the specific genes were found to be expressed in a consistent way in both qPCR and RNA-seq datasets indicating the compatibility of the observed transcriptional variations.

On the whole, the evidence in Figure 3 supports the idea that the CRISPR-mediated regulation of regulatory elements leads to gene-specific and genome-wide transcriptional alterations. These data points to the importance of enhancers and promoters in gene networks regulation and the efficacy of CRISPR-based tools in regulating gene expression in disease-relevant models.

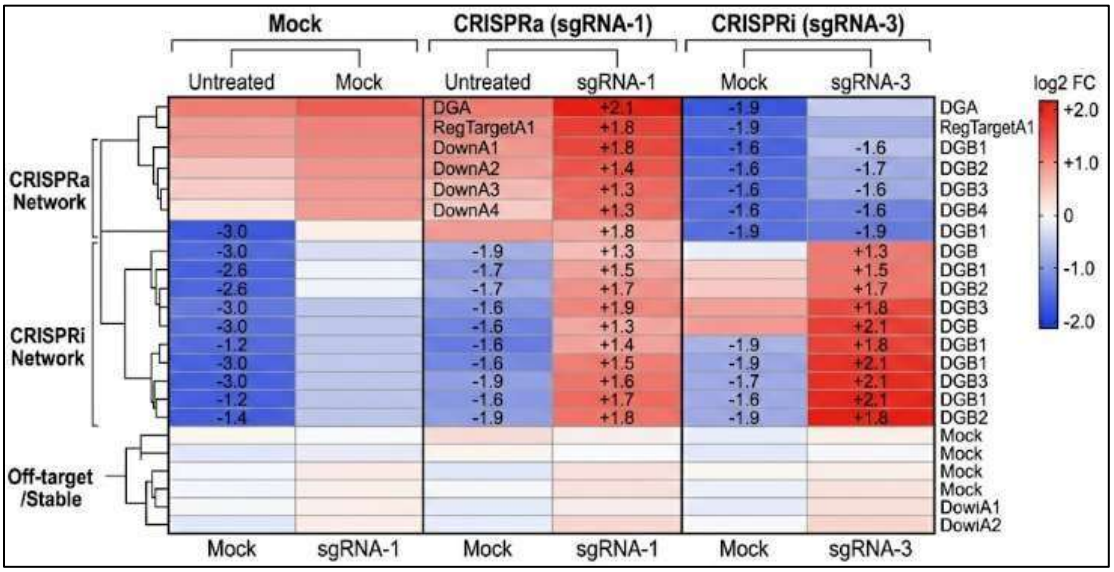

Figure 3. RNA-seq heatmap showing differential gene expression following CRISPR-mediated modulation of regulatory elements.

4.4 Functional Effects in Disease Models

Functional assays were performed to determine the biological effects of the modulation of regulatory elements in cell models of disease. Enhancer and promoter perturbation by CRISPR led to quantifiable cellular phenotypes, such as different cell proliferation and levels of apoptosis. There was a direct correlation between regulatory element activity and disease-associated cellular behavior, which is reflected by cells with altered gene expression profiles. These findings confirm the theory that disease-relevant phenotypes can be affected by the specific regulation of elements.

4.5 Off-target Analysis

The possibility of off-target effects with CRISPR based targeting was evaluated using computational prediction resources and experimental validation. It was found that there was little off-target activity and no significant non-target genomic changes were observed. This proves specificity of the designed sgRNAs and also justifies the credibility of CRISPR-based method used in this study.

5. DISCUSSION

The current paper reveals that non-coding regulation factor targeted modulation with CRISPR-Cas9 is a powerful method to study regulation of genes in disease models. We found that by targeting enhancers and promoters selectively we could reproducibly and locus-specifically change the expression of genes, which were then evident in quantifiable

changes in cellular phenotypes such as proliferation and apoptosis. These results substantiate the assumption that regulatory factors are vital in the regulation of gene activity and directly related to the disease-related behavior of cells. The noted similarity of molecular and functional results indicates the strength of CRISPR-based regulatory modulation as a functional genomics tool.

Our findings are in line with other earlier studies that had used CRISPR-based technologies to study the role of regulatory elements and enhancer promoter interactions. Previous studies have demonstrated that enhancers can be perturbed in a way that they can substantially change transcriptional outputs and modulate cellular phenotypes, especially in disease-relevant settings. CRISPR interference and activation systems are a more selective and reversible way to regulate gene expression compared to traditional methods of gene knockout which cause permanent changes in the genome. This work builds on these findings by incorporating specific regulation modulation and phenotypic validation, thus enhancing the relationship between non-coding genomic regions and disease processes. The biological significance of the identified regulatory elements is also improved with the help of built-in genomic annotations when choosing a target.

Although it has the above strengths, there are a number of limitations that should be taken into consideration. CRISPR efficiency variability and possibility of off-target effects can affect the precision of observed results, though our study has found that there are very few additional unintended edits. Moreover, the complexity of in vivo regulatory responses may not be quite represented by the use of in vitro disease models. Future research ought to aim at making better targeting specificity, further validation in more physiologically relevant systems like organoids or animal models and the therapeutic potential of CRISPR-mediated regulatory modulation. Developing a large-scale version of this technique to high-throughput screening systems might allow determining functional regulatory elements systematically and developing specific methods of gene regulation to cure any disease.

6. CONCLUSION

The paper has shown that targeted modulation of regulatory elements, using CRISPR, is both accurate and efficient in studying the regulation of genes in disease models. The highly specific regulation of enhancers and promoters resulted in large alterations in the expression of multiple genes and the ensuing cellular phenotypes, demonstrating the functional significance of non-coding genomic regions. Integration of genomic annotation with CRISPR-based tools provide a datasystematic structure in recognition and validation of disease-relevant regulatory elements. Notably, this system allows regulating genes in a controllable and reversible manner without altering the genome permanently, which is one of the main improvements made in comparison to classical gene editing technologies. In general, the findings suggest the promise of CRISPR-based regulatory modulation as a potent research method and as a promising approach to the development of therapeutic strategies in the future.

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