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Comprehensive analysis of splicing variants in corneal endothelial cells of patients with Fuchs endothelial corneal dystrophy

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Trinucleotide repeat (TNR) expansion in the transcription factor 4 *(TCF4)* gene represents the most prevalent genetic risk factor for Fuchs endothelial corneal dystrophy (FECD) and may cause dysfunction of splicing regulators. We investigated differential alternative splicing (DAS) events in corneal endothelial cells (CECs) from FECD patients with and without *TCF4* TNR expansion through RNA-Seq analysis. We identified distinct splicing profiles among control subjects, FECD patients with TNR expansion, and FECD patients without TNR expansion. Skipped Exon events constituted approximately 50% of all DAS events across all comparisons, with the remaining events distributed among alternative 3′ splice site, alternative 5′ splice site, mutually exclusive exon, and retained intron categories. Motif analysis in FECD patients with TNR expansion revealed several RNA-binding proteins, including *MBNL1*, as potential regulators of these splicing alterations. Computational analysis demonstrated that 34% of Skipped Exon events in the TNR expansion group significantly impacted protein structure. This comprehensive analysis revealed distinct alternative splicing signatures in FECD, particularly in cases with TNR expansion, suggesting a crucial role for aberrant splicing in FECD pathogenesis.

Fuchs endothelial corneal dystrophy (FECD) is a progressive, hereditary eye disease that affects the corneal endothelium and leads to vision impairment and potential blindness[1–3]. It affects approximately 4–11% of individuals and represents the leading indication for corneal transplantation[4]. Epidemiological studies have revealed that the clinical manifestation of FECD varies considerably, ranging from individuals who may remain asymptomatic throughout their lives to those who experience severe vision impairment requiring one or repeat transplants[5,6]. The pathogenesis of FECD is complex, involving both genetic and environmental factors[1–3].

One of the most common genetic factors associated with FECD is the expansion of trinucleotide repeats (TNR) in the transcription factor 4 *(TCF4)* gene[2,7]. This expansion is present in approximately 40–80% of FECD patients, making it the most prevalent genetic background for the disease[2]. The presence of TNR expansion in *TCF4* has led researchers to propose various hypotheses regarding its role in FECD pathogenesis. For example, the presence of intronic TNR expansion in FECD is reminiscent of myotonic dystrophies, suggesting potential mechanistic parallels between these disorders. In myotonic dystrophies, the expanded TNR sequence affects multiple RNA-binding proteins (RBPs) through distinct mechanisms. While the mutant RNA primarily sequesters muscleblind-like (MBNL) proteins, it also impacts other splicing regulators through different pathways, including CUG-binding protein elav-like family member 1 (CELF1; also referred to as CUGBP1), Staufen1, and heterogeneous nuclear ribonucleoprotein H (hnRNP H)[8,9]. The dysregulation of these splicing factors, particularly the sequestration of MBNL proteins, leads to widespread mis-splicing of target genes[10,11]. Studies utilizing a mouse model lacking functional RNA-binding activity of *MBNL1* protein have demonstrated alterations in hundreds of splicing events in muscle, heart, and brain tissues[12]. These findings underscore the extensive involvement of *MBNL* in the regulation of a wide array of splicing events and they support the critical role of *MBNL* in myotonic dystrophy[13].

Similar molecular mechanisms have been proposed for FECD, in which the expanded CTG repeat in *TCF4* may likewise sequester MBNL proteins, potentially contributing to disease pathogenesis through a disruption of

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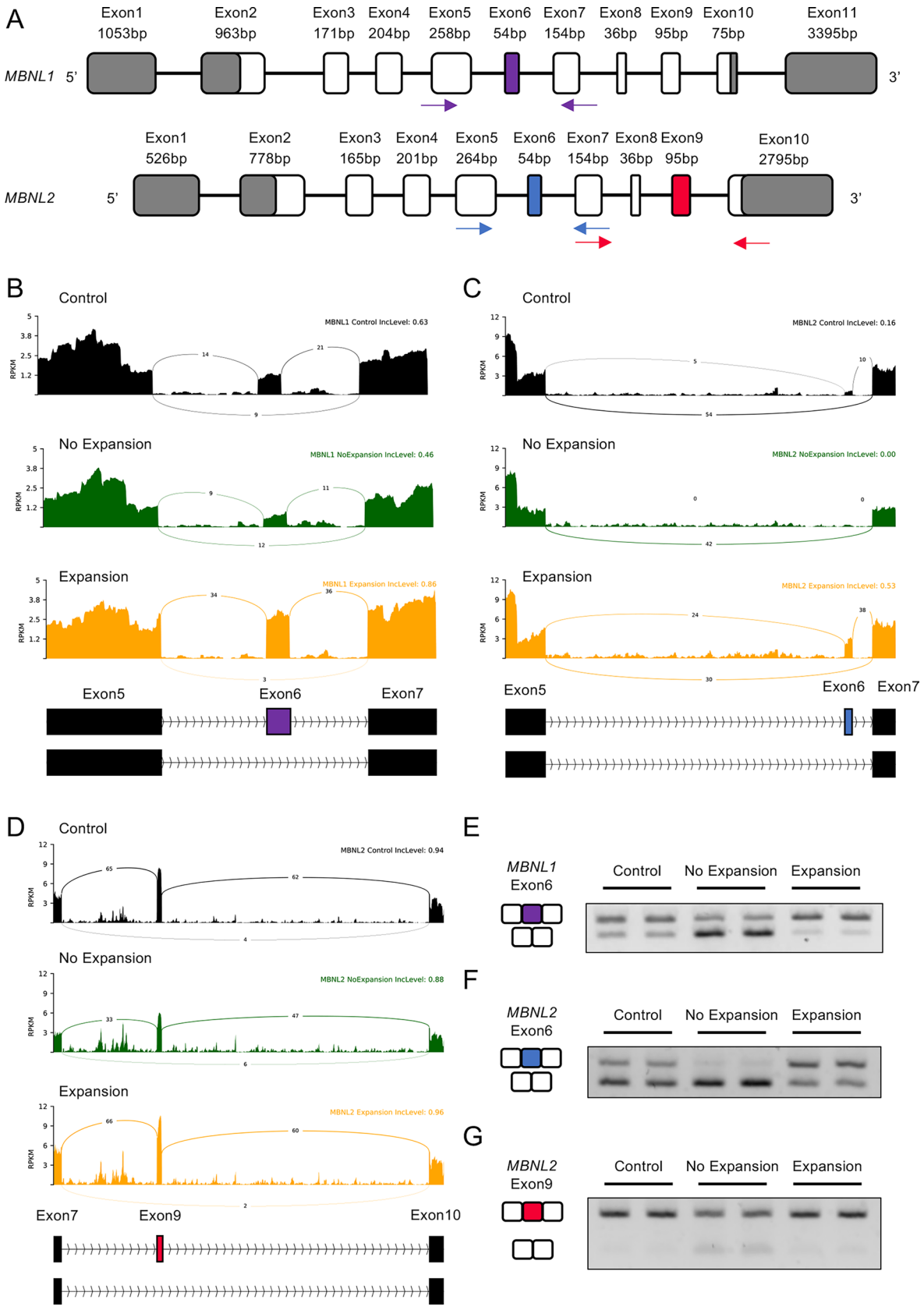

RNA processing[14,15]. Wieben and colleagues identified differential splicing in the *NUMA1*, *PPFIBP1*, *MBNL1*, *MBNL2*, *VEGFA*, and *FGFR2* transcripts in corneal endothelial cells (CECs) of patients with FECD harboring TNR expansion in *TCF4*, although larger numbers of potentially differential splicing events were also found[16]. Chu and colleagues also reported more than one thousand splicing event alterations in FECD with TNR expansion when compared with non-FECD control susbjects[17]. However, the genetic heterogeneity, varying disease stages,

◀**Fig. 1**. Alternative splicing events in *MBNL1* and *MBNL2* genes in Fuchs endothelial corneal dystrophy (FECD). (**A**) Schematic representation of the *MBNL1* and *MBNL2* gene structures. Previously reported alternatively spliced regions in FECD corneal endothelial cells are highlighted in purple, blue, and red. Gray regions indicate untranslated regions. Arrows indicate the positions of the RT-PCR primer pairs used for validation, summarized in (Supplementary Table 2). (**B**–**G**) Sashimi plots show increased inclusion of *MBNL1* exon 6 (**B**), *MBNL2* exon 6 (**C**), and *MBNL2* exon 9 (**D**) among the three groups. Representative gel images show RT-PCR validation results of splicing alterations in corneal endothelial cells (CECs) for increasing exon inclusion in the Expansion group compared to the Control and No Expansion group for (**E**) *MBNL1* exon 6, (**F**) *MBNL2* exon 6, and (**G**) *MBNL2* exon 9.

and racial diversity in FECD makes the accumulation of further evidence through validation studies crucial for obtaining a better understanding of the splicing events, as these may vary in healthy individuals as well as in the disease state.

The motivation for this study was to provide additional evidence that would elucidate the comprehensive landscape of altered splicing events in FECD, with the overall aim of offering new insights into the disease mechanism. To address the current knowledge gap, we analyzed splicing events in FECD using RNA-Seq data obtained from CECs of affected patients. Our study was designed to identify *MBNL*-regulated splicing events but also to uncover other splicing alterations that may contribute to FECD pathogenesis.

Results

Aberrant splicing in *MBNL1* and *MBNL2*

We analyzed alternative splicing events among three groups: 6 FECD patients with TNR expansion (Expansion group), 4 FECD patients without TNR expansion (No Expansion group), and 7 non-FECD controls (Control group). We first investigated the splicing patterns of *MBNL1* and *MBNL2* genes, as previous studies have demonstrated their splicing dysregulation in FECD patients carrying TNR expansions in *TCF4*[16,18]. MBNL proteins are known to regulate alternative splicing of their own pre-mRNAs. In both myotonic dystrophy and FECD with TNR expansions, altered MBNL function leads to increased inclusion of *MBNL1* exon 6, *MBNL2* exon 6, and *MBNL2* exon 9 compared to controls, suggesting a disruption in the autoregulatory splicing mechanism[13,16,18]. This enabled corroboration of these findings and validated the consistency for our RNA-Seq data. The gene sequences of *MBNL1* and *MBNL2* were examined with a particular focus on regions where splicing alterations had been previously identified (Fig. 1A). In *MBNL1*, exon 6 demonstrated increased inclusion in the Expansion group compared to both the Control and No Expansion groups (Fig. 1B). A similar pattern was observed for exon 6 of *MBNL2*, with elevated inclusion in the Expansion group relative to both Control and No Expansion groups (Fig. 1C). For exon 9 of *MBNL2*, increased inclusion was observed in the Expansion group compared to the No Expansion group, although the inclusion pattern was comparable between the Expansion and Control groups (Fig. 1D).

To confirm these aberrant splicing events, we further validated the splicing alterations using RT-PCR. This analysis was performed to verify the splicing alterations observed in *MBNL1* exon 6 (Fig. 1E), *MBNL2* exon 6 (Fig. 1F), and *MBNL2* exon 9 (Fig. 1G). The results from the RT-PCR experiments corroborated the findings from the Sashimi plots, demonstrating consistent splicing alterations in the Expansion group across these exons. Original gels are presented in (Supplementary Fig. 1).

Identification of differential alternative splicing (DAS) events

We subsequently evaluated DAS events occurring across all genes detected by RNA-Seq. The principal component analysis (PCA) revealed distinct clustering patterns in splicing profiles among the control, no expansion, and expansion groups. Each cohort formed discrete clusters, indicating substantial differences in alternative splicing patterns (Fig. 2A). Hierarchical clustering analysis corroborated these findings, demonstrating distinct patterns of splicing events across the groups (Fig. 2B).

Comparison of the expansion and control groups for all genes in RNA-Seq data yielded 1,816 DAS events among five patterns (Skipped Exon (SE): 974, Alternative 3' Splice Site (A3SS): 161, Alternative 5' Splice Site (A5SS): 155, Mutually Exclusive Exons (MXE): 200, and Retained Intron (RI): 326). The Expansion versus No Expansion group comparison identified 809 DAS events (SE: 404, A3SS: 84, A5SS: 91, MXE: 74, RI: 156). Lastly, the No Expansion versus Control group comparison revealed 2,299 DAS events (SE: 1,131, A3SS: 202, A5SS: 171, MXE: 239, RI: 556) (Fig. 3A). The distribution of event types for the Expansion versus Control comparison was as follows: SE: 53.6%, A3SS: 8.9%, A5SS: 8.5%, MXE: 11.0%, and RI: 18.0% (Fig. 3B). For the Expansion versus No Expansion comparison, the distribution was SE: 49.9%, A3SS: 10.4%, A5SS: 11.3%, MXE: 9.2%, and RI: 19.3% (Fig. 3C). Similarly, for the No Expansion versus Control comparison, events were distributed as SE: 49.2%, A3SS: 8.8%, A5SS: 7.4%, MXE: 10.4%, and RI: 24.2% (Fig. 3D). SE was consistently the most frequent splicing event type across all group comparisons.

To determine whether DAS events were specifically induced by TNR expansion in *TCF4* or by other factors such as unknown genetic backgrounds in the No Expansion group or secondary changes associated with FECD development, we compared DAS events identified from two distinct analyses: Expansion versus Control and No Expansion versus Control. Venn diagrams revealed overlapping events as follows: SE: 383, A3SS: 51, A5SS: 39, MXE: 41, and RI: 124. Although these events overlapped between the Expansion and No Expansion groups, higher numbers of unique DAS events were found in both the Expansion versus Control and No Expansion versus Control comparisons (Fig. 4).

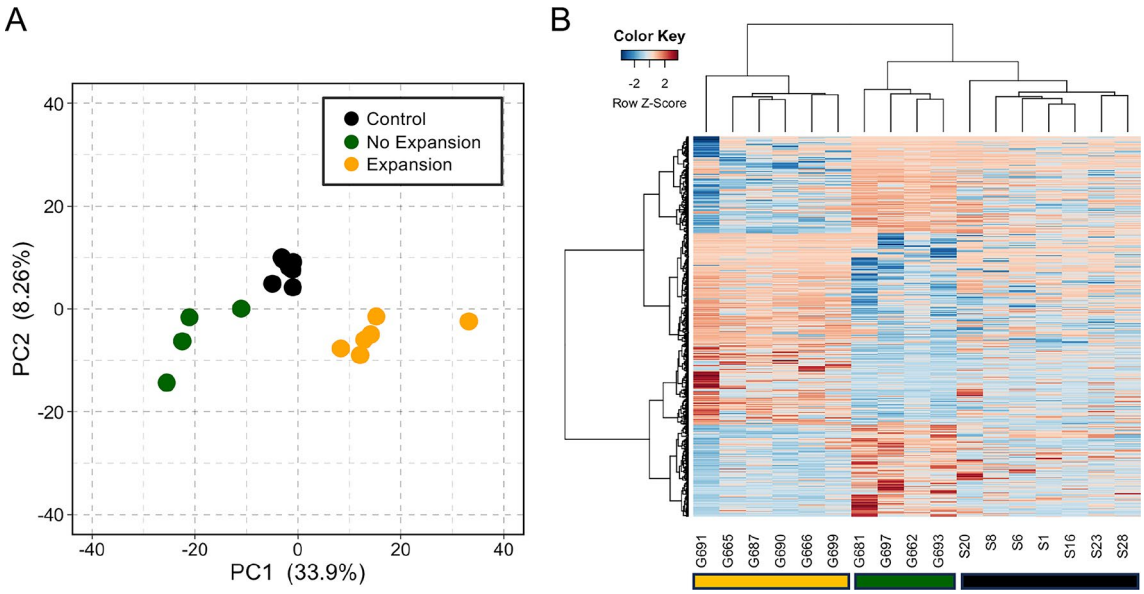

Fig. 2. Global analysis of alternative splicing patterns in Fuchs endothelial corneal dystrophy (FECD). (**A**) Principal component analysis (PCA) of alternative splicing events across the study groups. Control (black), No Expansion (green), and Expansion (yellow) samples are plotted in PC1 versus PC2 space. The percentage of total variance explained by each principal component is indicated on the respective axes. (**B**) Hierarchical clustering heatmap depicting relative inclusion levels of differentially alternatively spliced (DAS) events across Control (black), No Expansion (green), and Expansion (yellow) groups, demonstrating distinct splicing signatures between FECD patients (No Expansion and Expansion groups) and non-FECD controls.

Identification of RBPs

We explored the regulatory mechanisms underlying the differential splicing patterns by performing motif analyses on SE events that differed between the Expansion and No Expansion groups. Through rMATS analysis, we identified 404 significant differential SE events between the Expansion and No Expansion groups (Fig. 3A). Of these, 184 events demonstrated increased exon inclusion, while 220 events showed increased exon skipping in the Expansion group compared to the No Expansion group. To better understand the regulatory mechanisms underlying these splicing changes, we performed motif enrichment analysis to identify potential RBPs associated with each splicing pattern separately. Among the 404 total differential SE events identified, 184 events showed increased inclusion while 220 events exhibited increased skipping in the Expansion group compared to the No Expansion group. Analysis of the 184 inclusion events revealed enrichment of binding motifs for several RBPs, including HuR, PTB, SFPQ, SNRPA, and YBX2, as illustrated in the heatmap (Fig. 5A). For the 220 skipping events, we identified potential binding sites for 11 distinct RBPs, with *MBNL1* emerging as a key regulatory factor in these events (Fig. 5B).

Functional enrichment of splicing variants

A total of 404 SE events showing splicing alterations between the No Expansion and Expansion groups were analyzed to determine their potential impact on the resulting proteins. Overall, 34% of the SE events affected the resulting proteins, while the remaining 66% had no impact on the protein. Among these 34% events, 85% were located in protein domains, 10% affected residues, and 5% influenced linear motifs (Fig. 6A). Enrichment analysis was performed on the genes harboring the 34% of exons that were predicted to impact the proteins. Reactome pathway analysis revealed that these exons were associated with pathways such as the immune system, adaptive immune system, signaling by receptor tyrosine kinases, and *FGFR1* ligand binding and activation (Fig. 6B). KEGG pathway analysis indicated associations with pathways including the MAPK signaling pathway, PI3K-Akt signaling pathway, Ras signaling pathway, focal adhesion, and apoptosis (Fig. 6C).

Discussion

This study examined splicing events in the CECs of patients with FECD and revealed distinct splicing patterns among Expansion, No Expansion, and Control groups. As anticipated, expansion patterns diverged from both control and No Expansion groups, although the No Expansion group also unexpectedly exhibited splicing patterns that differed from the Control group. Among the alternative splicing events observed in the Expansion group, approximately half were Skipped Exons compared to the No Expansion and Control groups. Among 184 events involving exon skipping in which typically included exons were skipped in the Expansion group, 11 splicing regulatory factors influencing these skipped exons, including *MBNL1*, were identified. Current research supports the hypothesis that TNR expansion-induced *MBNL* dysregulation leads to splicing abnormalities in multiple genes, potentially contributing to corneal endothelial dysfunction.

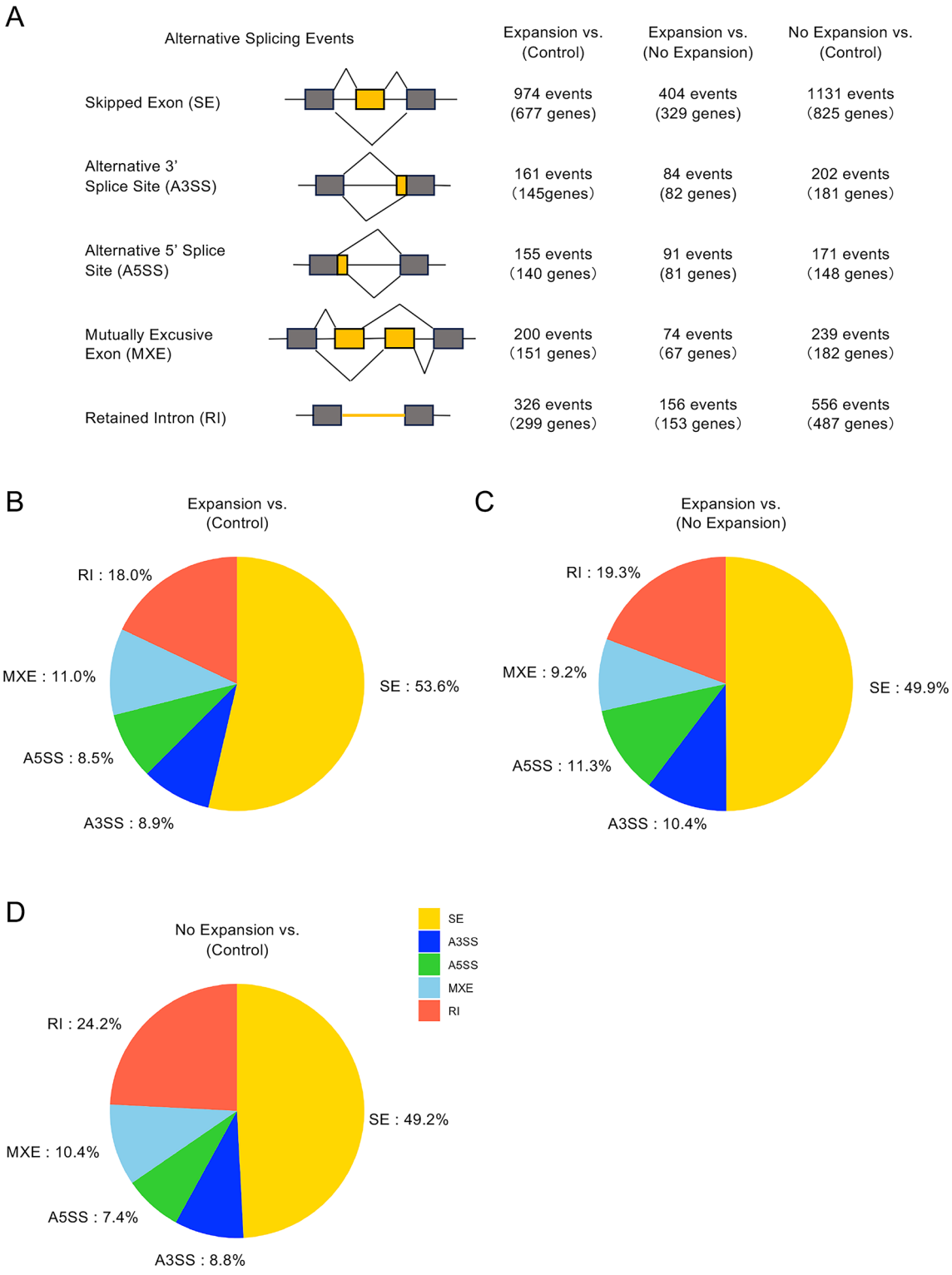

Fig. 3. Analysis of differential alternative splicing (DAS) events in Fuchs endothelial corneal dystrophy (FECD). (**A**) Schematic diagram of five splicing patterns of an alternative splicing event. Distribution of significant DAS events and affected genes across five splicing patterns: skipped exon (SE), alternative 3' splice site (A3SS), alternative 5' splice site (A5SS), mutually exclusive exon (MXE), and retained intron (RI). Significance criteria: FDR < 0.05 and |IncLevelDifference| > 0.2. (**B**–**D**) Pie charts showing the relative proportions of five types of differential splicing patterns (SE, A3SS, A5SS, MXE, and RI) identified between Expansion versus Control groups (**B**), Expansion versus No Expansion groups (**C**), and No Expansion versus Control groups (**D**). The percentages represent the proportion of each splicing pattern type among all DAS events detected in each comparison.

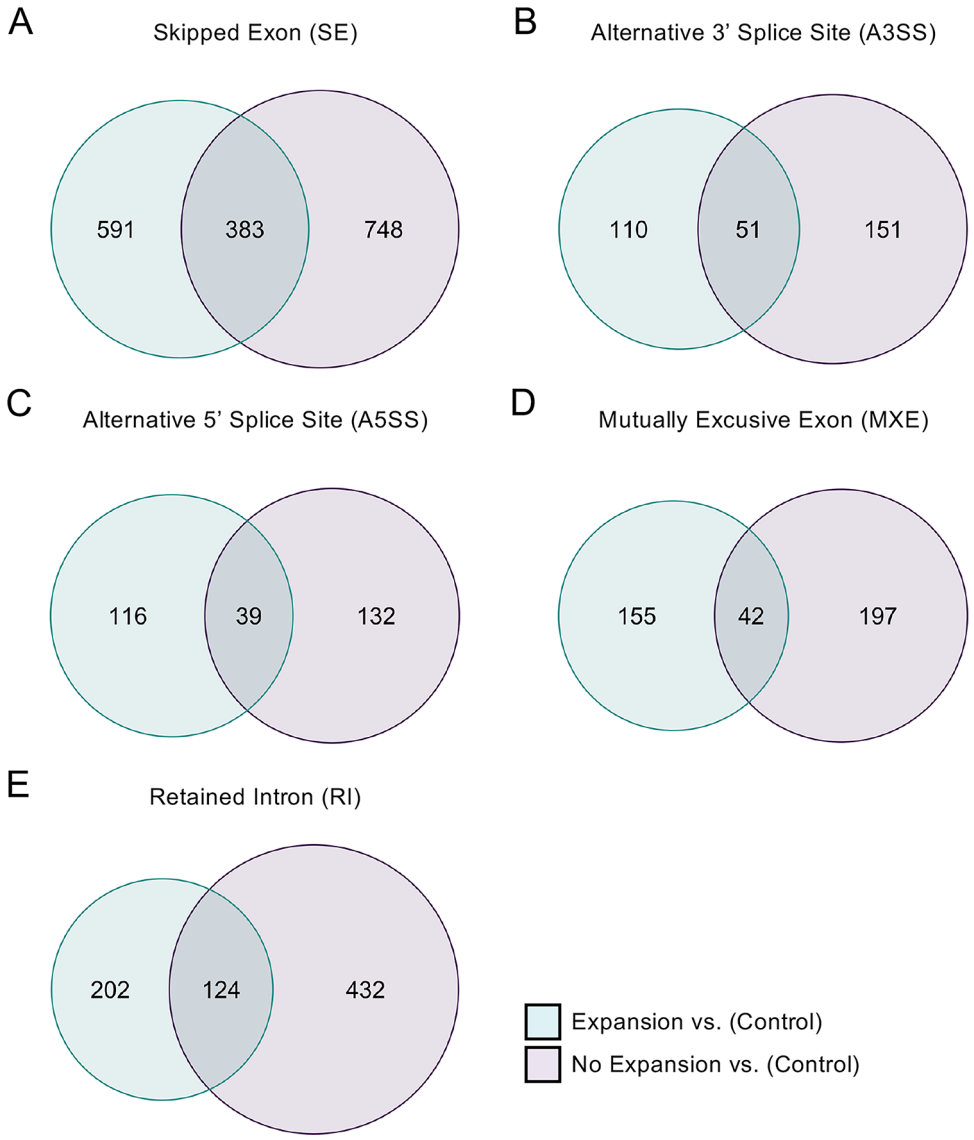

Fig. 4. Comparison of alternative splicing events between Fuchs endothelial corneal dystrophy (FECD) groups. Venn diagrams comparing differential alternative splicing (DAS) events identified between two distinct analyses: Expansion versus Control and No Expansion versus Control. The blue circles represent DAS events specific to the expansion versus control comparison, while purple circles indicate DAS events specific to the No Expansion versus Control comparison. Overlapping regions show shared DAS events between both comparisons. Separate diagrams are shown for each splicing pattern: (**A**) skipped exon (SE), (**B**) alternative 3' splice site (A3SS), (**C**) alternative 5' splice site (A5SS), (**D**) mutually exclusive exon (MXE), and (**E**) retained intron (RI). All DAS events met significance criteria of FDR < 0.05 and |IncLevelDifference| > 0.2.

Alternative splicing is a fundamental mechanism that generates proteomic diversity from a limited genome, thereby enabling the generation of multiple protein isoforms from a single gene. This process fosters cellular heterogeneity and tissue-specific functions, allowing precise responses to developmental and environmental cues. Its evolutionary conservation underscores its importance in biological adaptation and resilience[19–21]. However, while alternative splicing plays a crucial role in generating biological diversity, aberrations in this process can lead to pathological conditions[22].

Splicing errors can result in the loss of protein function, production of deleterious protein isoforms, or disruption of cellular homeostasis[23]. These abnormalities have been implicated in a wide range of diseases, including neurodegenerative disorders, cancers, and genetic syndromes[24–26]. These types of aberrations may arise from mutations in splice sites, dysregulation of splicing factors, or dysfunction of the spliceosome machinery[27,28]. The significance of alternative splicing in both normal physiology and pathological states highlights its importance as a subject of ongoing research in molecular biology and medicine. Understanding the mechanisms and consequences of splicing alterations may provide valuable insights into disease pathogenesis and potential therapeutic strategies.

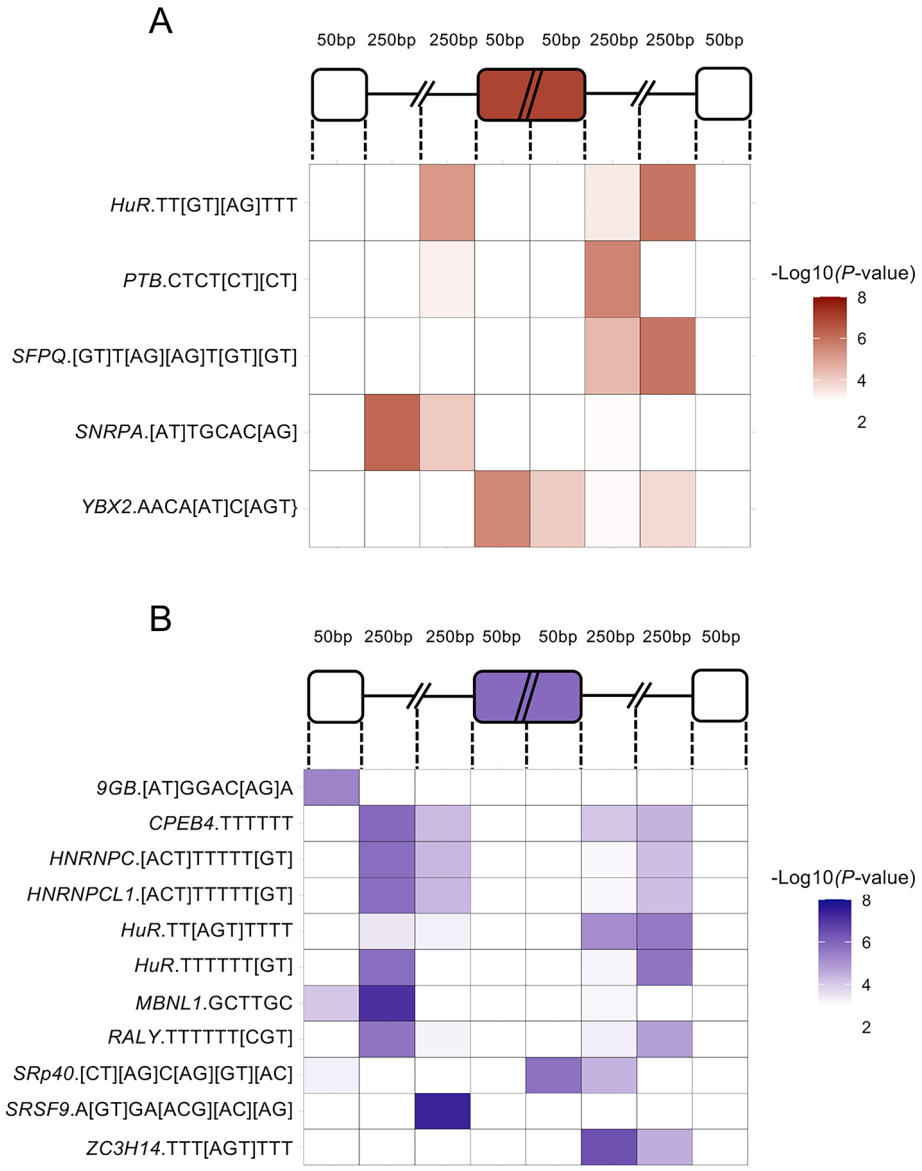

Fig. 5. Motif enrichment analysis of RNA-binding proteins (RBPs) in Skipped Exon (SE) patterns between expansion and no expansion groups. The heatmaps show the motif enrichment analysis for RBPs potentially binding near the introns and exons of genes with altered splicing patterns. The left side of each heatmap lists RBPs, with their corresponding binding motifs shown beside them. The color intensity of the heatmap indicates the statistical significance (*P*-value), with darker shades representing more significant enrichment. The schematic diagram depicts two types of SE events: (**A**) exons that undergo increased skipping and (**B**) exons that show increased inclusion. For each event type, we analyzed the following regions: 50 bp from both ends of the differentially regulated exon, 50 bp from the edges of adjacent upstream and downstream exons, and 250 bp from both ends of the flanking introns. The analysis focuses on exons included and skipped in the Expansion group compared to the No Expansion group, both within the context of SE patterns.

In FECD, splicing abnormalities have become a significant focus in research into its pathogenesis. The discovery of CTG trinucleotide repeat expansions in the *TCF4* gene as a major genetic mutation led to investigations into RNA-mediated toxicity mechanisms. Studies have revealed that expanded CUG-containing transcripts form nuclear RNA foci sequestering splicing factors, particularly *MBNL* proteins[2,14,18]. RNA-Seq analyses have demonstrated widespread aberrant splicing events correlated with CTG18.1 expansion status[16,17]. Our study provides additional evidence for aberrant splicing events in numerous genes with multiple alternative splicing patterns in patients with *TCF4* TNR expansion. Analysis of exon skipping regulators revealed 11 genes, including *MBNL1*, aligning with the hypothesis that *MBNL1* impairment, induced by TNR expansion, precipitates aberrant splicing. Furthermore, these findings suggest a potential involvement of other splicing modulators in patients with *TCF4* repeat expansions. Further research is needed to elucidate which splicing factors induce pathological aberrant splicing events that result in FECD.

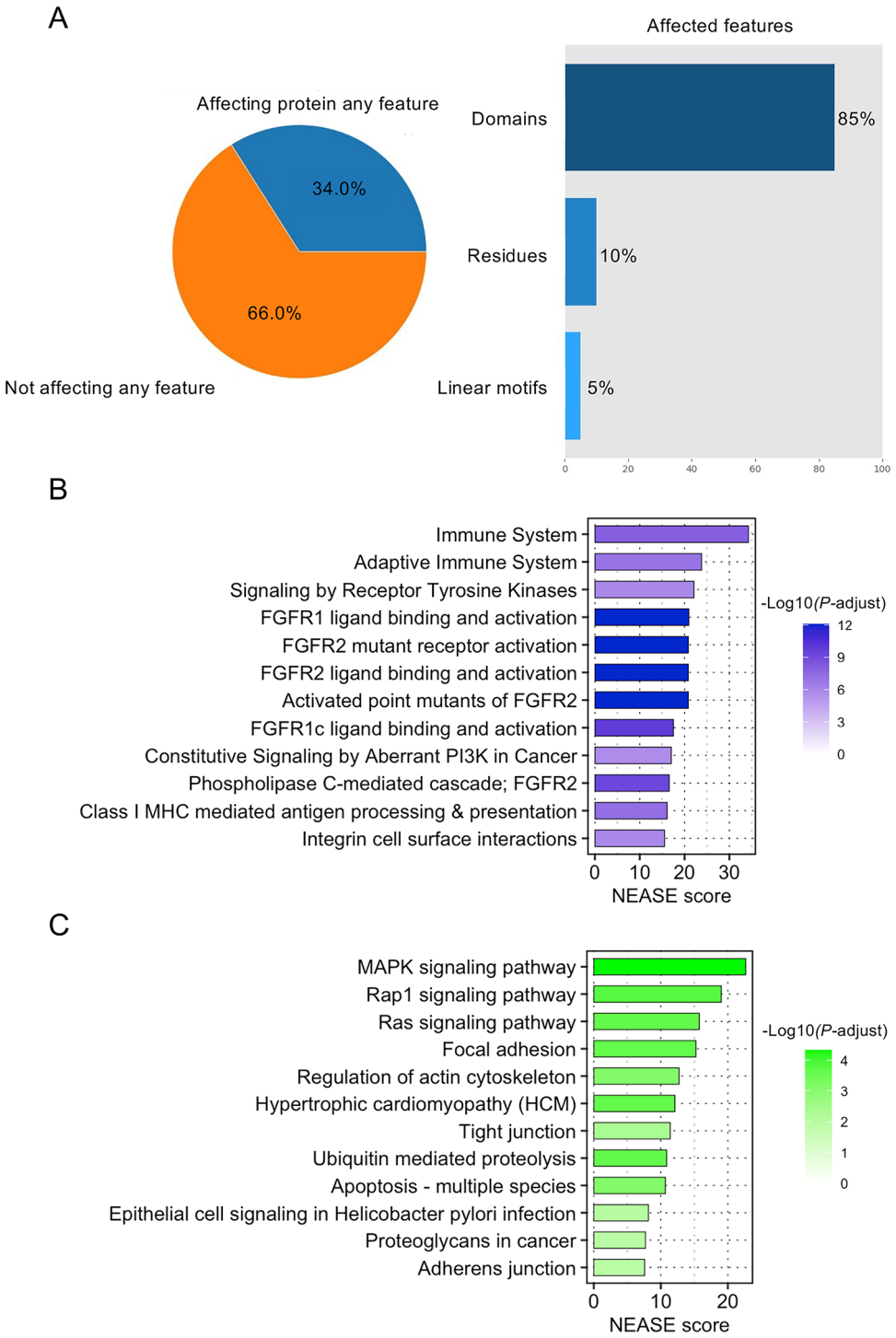

Fig. 6. Functional and pathway analysis of skipped exon (SE) patterns altered between the expansion and no expansion groups. (**A**) The pie chart shows the proportion of exons, identified through SE patterns, that affect protein structure or function when splicing changes occur. The bar graph further categorizes these exons into domains, residues, and linear motifs, representing their relative proportions. (**B**) Reactome pathway analysis of genes with SE events that were altered in the Expansion group compared to the no expansion group and affect protein structure or function. (**C**) KEGG pathway analysis for the same set of genes. In both the Reactome and KEGG pathway analyses, the top 12 pathways with the highest NEASE scores are displayed. The color intensity represents the statistical significance (adjusted *P*-value).

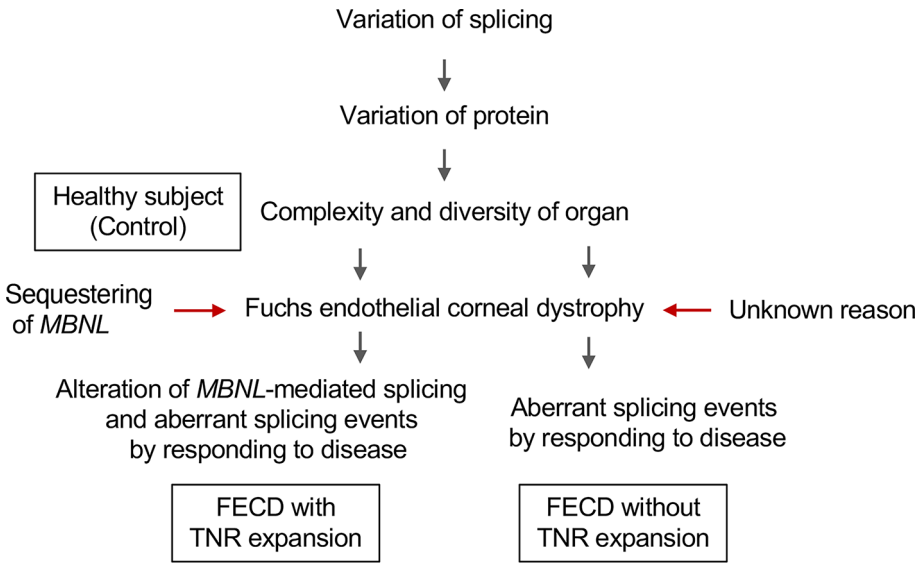

Fig. 7. Proposed model of splicing aberrations based on *TCF4* Trinucleotide repeat (TNR) expansion status. Schematic representation of splicing aberration mechanisms in Fuchs endothelial corneal dystrophy (FECD) based on the *TCF4* TNR expansion status. In FECD patients with TNR expansion, disease progression is initiated by *MBNL* sequestration, which leads to widespread aberrant splicing patterns. By contrast, FECD patients without TNR expansion exhibit splicing alterations that likely occur as a secondary response to disease progression triggered by currently unknown pathogenic mechanisms.

The observed differential splicing patterns between the No Expansion and Control groups, while unexpected, warrant careful interpretation within the context of disease pathophysiology. Our analysis revealed that the number of DAS events was paradoxically higher in the No Expansion versus Control comparison than in the Expansion versus Control comparison. However, this quantitative difference should be interpreted cautiously, as aberrant splicing events can function as both etiological factors and downstream effects of pathological processes[29]. We propose that the distinct splicing patterns observed in these groups may reflect different underlying molecular mechanisms. In the Expansion group, splicing alterations likely arise from a combination of *MBNL1* sequestration by expanded *TCF4* TNR and secondary effects of disease progression. Conversely, in the No Expansion group, the splicing changes may primarily result from disease progression effects, potentially in conjunction with yet unidentified genetic factors (Fig. 7). The toxic effects of *TCF4* TNR expansion-mediated alterations in MBNL-dependent splicing are likely central to the pathogenesis in the Expansion group. Therefore, future research should focus not only on the quantity of DAS events but also on identifying and characterizing specifically toxic splicing alterations. This distinction is crucial for understanding the pathogenic mechanisms and developing targeted therapeutic strategies. This hypothesis highlights the complex interplay between genetic predisposition, molecular mechanisms, and disease progression in the pathogenesis of this condition. Further studies are needed to elucidate the precise mechanisms underlying these differential splicing patterns and their respective contributions to disease etiology and progression.

Mis-spliced *TCF4* transcripts were identified in patients harboring TNR expansion, potentially leading to pathogenic *TCF4* isoforms or altered expression levels[30–32]. While dysregulated *TCF4* expression has been proposed as a pathological mechanism, some studies have reported inconsistent results, likely due to qPCR limitations given numerous isoforms of *TCF4*. Indeed, RNA-Seq analyses revealed differential expression of multiple *TCF4* isoforms in FECD patients with TNR expansions, suggesting TNR contribution to dysregulated *TCF4* expression[30,32,33]. Sirp et al. demonstrated downregulation of *TCF4* isoforms from alternative 5' exons near TNR, while other isoforms were upregulated[30]. Long-read RNA-Seq confirmed lower expression of *TCF4* isoforms downstream of TNR in FECD patients[34]. We previously identified *TCF4-277* as a commonly dysregulated isoform in FECD with TNR expansion[32]. However, whether *MBNL1* abnormalities cause *TCF4* splicing defects in FECD remains unclear, and further investigation is anticipated to clarify this question.

A limitation of this study is the uncertainty regarding the extent to which *MBNL1* sequestration due to TNR expansion contributes to the numerous splicing abnormalities. Furthermore, the potential involvement of other splicing factors cannot be excluded. One noteworthy point is that diverse splicing events also occur in the healthy corneal endothelium. The fact that splicing aberrations can be both etiological factors and consequences of disease processes complicates the isolation and identification of specific splicing abnormalities induced by TNR expansion that directly contribute to FECD pathogenesis. However, one pertinent observation is that in other fields, such as oncology, several therapeutic agents targeting splicing abnormalities have been developed and are currently undergoing clinical trials[35,36]. This precedent suggests that targeting splicing aberrations in FECD could potentially offer a novel therapeutic approach. Therefore, further investigation into splicing mechanisms in FECD is warranted, as it may lead to the development of innovative treatment strategies.

Methods

Ethics statement

This investigation was conducted in accordance with the ethical standards outlined in the Declaration of Helsinki. The study protocol received approval from the ethics committees of three institutions: the Ethics Committee for Scientific Research Involving Human Subjects at Doshisha University (reference number: 20009), the Institutional Review Board of Kyoto Prefectural University of Medicine (reference number: ERB-G-73), and the Ethics Committee of Friedrich-Alexander Universität Erlangen-Nürnberg (reference number: 140_20 B). Participants at Friedrich-Alexander Universität Erlangen-Nürnberg provided informed consent for the collection of peripheral blood samples and Descemet's membranes, including CECs. All procedures in this study were executed in compliance with the guidelines set forth by these ethical committees.

Acquisition and processing of RNA-Seq data

This study utilized RNA-Seq data from CECs of both non-FECD controls and FECD patients, as we previously reported[37,38]. Raw fastq files were accessed from the DNA Data Bank of Japan Sequence Read Archive (accession IDs: DRA010237 for non-FECD and DRA015078 for FECD subjects). Sample information from these RNA-Seq data is available in (Supplementary Table 1). Previous studies utilized peripheral blood samples to classify FECD patients based on the presence or absence of TNR expansion in *TCF4*[37,38]. The threshold of 50 repeats was chosen based on its established association with FECD pathogenesis[7]. In our cohort, all No Expansion samples had fewer than 40 repeats, while Expansion samples had 50 or more repeats, with no samples falling within the intermediate range of 40–50 repeats. In the present study, we investigated RNA-Seq data of CECs derived from these previously characterized FECD patients with known TNR expansion status. Data processing commenced with elimination of low-quality reads from raw fastq files using fastp software (v 0.20.0)[39]. Subsequently, the processed reads were aligned to the human reference genome (Homo_sapiens.GRCh38.111) using STAR aligner (v 2.7.10a)[40]. Gene annotations were based on the Ensembl annotation file (GRCh38.111.gtf).

Identification and analysis of DAS events

The analysis of DAS events was performed using rMATS-turbo (v4.3.0)[41,42]. Five types of alternative splicing events were examined: SE, A3SS A5SS, MXE, and RI. Statistical significance was determined by adjusting *P*-values using the Benjamini-Hochberg method to control for the false discovery rate (FDR). DAS events were considered significant if they met the following criteria: FDR < 0.05 and |IncLevelDifference| > 0.2. The significant DAS events identified from the comparisons of the No Expansion group with the Control group, and the Expansion group with the Control group, were displayed as Venn diagrams using the "VennDiagram" package (v1.7.3) in R (version 4.4.1) to visualize the overlap between these datasets. Visualization of specific genes with significant differences was accomplished using rmats2sashimiplot (v3.0.0).

To identify potential splicing regulators, we applied rMAPS2 (v2.2.0)[43,44] to assess the enrichment of RBPs motifs near alternatively spliced exons in DAS events identified by rMATS-turbo. This analysis was performed on both exon inclusion and skipping events, aiming to elucidate the regulatory mechanisms underlying the splicing alterations observed in FECD. The results were also visualized using a heatmap generated with the "ggplot" function from the "ggplot2" package (v3.5.1) in R.

Assessment and confirmation of data profile

PCA was performed using the "prcomp" function from the "base" package in R to assess the overall distribution of samples based on their splicing patterns. Heatmap analysis was conducted using the "heatmap.2" function from the "gplots" package (v3.1.3.1) in R. Hierarchical clustering was applied using the "ward.D2" option in the "hclust" function from the "stats" package in R to group samples and splicing events based on similarity. We visualized the distribution of different types of alternative splicing events by generating a pie chart based on the counts of each splicing event type (SE, A3SS, A5SS, MXE, and RI) across all samples by using the "ggplot" function from the "ggplot2" package in R.

Domain and pathway enrichment analysis in DAS events

NEASE (v1.3)[45] software was used to assess whether the exons involved in DAS events affect the resulting protein structure. This analysis focused on the impact of these exons on protein domains, residues, or linear motifs. Splicing events were classified based on their potential to disrupt or alter these functional features, and the proportion of affected events in each category was calculated. For DAS events predicted to impact protein domains or functional features, pathway enrichment analysis was performed within the NEASE analysis using Reactome[46] and KEGG databases[47,48]. The results were ranked based on NEASE scores, with significantly enriched pathways identified by adjusted *P*-values < 0.05, thereby providing insights into the broader biological consequences of the splicing alterations.

Validation of splicing alterations with RT-PCR

Corneal endothelial tissue of patients with FECD was obtained during corneal transplantation. For controls, we utilized residual healthy corneal endothelium from non-FECD donor corneas collected during corneal transplantation procedures. The *TCF4* TNR status of all samples was previously characterized in our studies[49], confirming that none of the non-FECD donor corneas harbored TNR expansion. Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Valencia, CA). cDNA was synthesized from the extracted RNA using the ReverTra Ace -α- (Toyobo, Osaka, Japan) according to the manufacturer's instructions. PCR amplification was performed using KOD FX Neo (Toyobo) with the following conditions: 90 °C for 2 min (1 cycle), 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 15 s (32 cycles), followed by a final extension at 68 °C for 5 min. The PCR primers for *MBNL1* and *MBNL2* were designed based on previously published studies (Supplementary Table 2)[50,51].

The amplification products were separated on a 2.5% agarose gel in TBE running buffer and imaged using the Amersham Imager 600 (Cytiva, Tokyo, Japan).

Data availability

The RNA-Seq datasets analyzed during the current study are available in the DNA Data Bank of Japan Sequence Read Archive repository with the accession IDs: DRA010237(https://ddbj.nig.ac.jp/search/entry/sra-submission/DRA010237) (non-FECD controls)[37] and DRA015078(https://ddbj.nig.ac.jp/search/entry/sra-submission/DRA015078) (FECD subjects)[38]. Sample information is available in (Supplementary Table 1).

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Author contributions

T.Y., Y.T., M.N., N.K., and N.O. conceived and designed the study. T.Y., Y.T., and M.N. performed the experiments. T.Y. and N.O. drafted the article, and all authors revised the paper and approved the final version to be published.

Declarations

Competing interests

Naoki Okumura and Noriko Koizumi are co-founders of ActualEyes Inc., which is currently developing a pharmaceutical therapy for treating Fuchs endothelial corneal dystrophy. However, these affiliations did not influence the design, execution, interpretation, or reporting of the research presented in this paper. Other authors do not have a competing financial interest.

Additional information

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