

AAV2-mediated intravitreal delivery of exon-specific U1 snRNA rescues optic neuropathy in familial dysautonomia

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Familial dysautonomia (FD) is a rare autosomal recessive neurodegenerative disorder caused by a splicing mutation in the *ELP1* gene. It predominantly affects the sensory and autonomic nervous systems, with progressive vision loss due to optic neuropathy being a universal and debilitating symptom. Retinal pathology in FD involves progressive thinning of the retinal nerve fiber layer (RNFL), resulting from the degeneration of retinal ganglion cells (RGCs). Notably, FD-associated vision loss has a postnatal onset, offering a critical window for therapeutic intervention before severe visual impairment develops in adolescence. Currently, no approved treatments exist to prevent or reverse vision loss in FD. In this study, we present a novel RNA-based therapeutic approach targeting *ELP1* pre-mRNA splicing in the retina. We engineered exon-specific U1 small nuclear RNAs (ExSpeU1s) to enhance inclusion of exon 20 in the mutant *ELP1* transcripts in the retina, thereby restoring full-length *ELP1* expression. Delivery of ExSpeU1 via adeno-associated virus serotype 2 (AAV2) to the retina improved *ELP1* splicing, rescued RGC loss, and visual function in an FD mouse model. These findings highlight ExSpeU1-mediated splicing correction as a promising therapeutic approach for treating optic neuropathy in FD, offering potential to preserve vision and improve quality of life for patients.

INTRODUCTION

Familial dysautonomia (FD) is a rare congenital sensory and autonomic neuropathy characterized by symptoms such as reduced sensitivity to pain and temperature, early feeding difficulties, cardiovascular instability, and gait ataxia.¹ The average life expectancy of affected individuals is approximately 40 years.^{1–5} FD is most common in individuals of Ashkenazi Jewish ancestry (carrier frequency ~1:32; ~1:17 in those of Polish descent)⁶ and classified as an ultra-rare disease with a prevalence <1/50,000.⁷ In addition to their sensory and autonomic deficits, FD patients also suffer from progressive blindness, which has an onset in the second decade and severely

impacts their quality of life.^{8–10} Major ocular defects include decreased visual acuity, loss of central vision, and temporal optic nerve pallor.^{8–11} The retinal abnormalities observed in FD patients are similar to other well-characterized optic neuropathies such as Leber hereditary optic neuropathy (LHON) and dominant optic neuropathy including reduction of retinal nerve fiber layer (RNFL) due to the death of retinal ganglion cells (RGCs).^{8–10} FD patients suffer from progressive optic neuropathy beginning as early as five years of age^{8–10} with a slowed progression until approximately 15–20 years, followed by a significant visual decline resulting in patients becoming visually impaired by their second decade of life.¹¹ Since FD patients have diminished proprioception, this visual loss severely affects patient mobility, leading to frequent falls, further compromising their quality of life. The relatively slow progression of visual decline in FD provides a therapeutic window during which early intervention could significantly preserve vision and improve quality of life.

FD is caused by a T to C nucleotide change in the 5' splice site of intron 20 of the elongator acetyltransferase complex subunit 1 (*ELP1*, also known as *IKBKAP*). This mutation leads to skipping of *ELP1* exon 20, resulting in tissue-specific mis-splicing and reduction of *ELP1* protein predominantly in the central and peripheral nervous system.^{2,3,5} *ELP1* plays a major role in catalyzing protein translation, tRNA modification, transcriptional elongation, and cytoskeletal

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remodeling.^{12–14} We have recently generated a phenotypic humanized FD mouse model, *TgFD9;Elp1^{Δ20/flox}*, which carries the human *ELP1* transgene with the FD major mutation and expresses very low amounts of the endogenous *Elp1* gene.^{15,16} This mouse reveals progressive loss of the RNFL and ganglion cell layer (GCL), as observed in FD patients, while recapitulating the same tissue-specific skipping of *ELP1* exon 20.^{15–17}

Several therapeutic strategies show promise in rescuing RGC loss in mouse models of FD.^{5,17–22} Splice modulatory compounds (SMCs), which target defective splicing, have been shown to restore RNFL and ganglion cell inner plexiform layer (GCIPL) thickness and preserve RGC counts in FD mice. SMC offers the advantage of systemic delivery, potentially addressing both retinal and systemic defects, and can be administered with relative ease, most likely through oral dosing.¹⁷ However, their off-target effects must be carefully evaluated before clinical translation. Gene replacement therapy for *ELP1* has also been shown to rescue RGC loss in FD, but challenges remain, including potential toxicity from *ELP1* overexpression, which could disrupt protein turnover and cellular homeostasis, and the large size of the *ELP1* gene, which limits precise expression control in target retinal cells. An alternative approach involves antisense oligonucleotides (ASOs) as splice modifiers. ASOs have been shown to significantly correct *ELP1* splicing in transgenic FD mice lacking overt phenotypic defects. However, their efficacy has yet to be tested in a phenotypic model of FD.

U1 small nuclear RNA (U1 snRNA) is a core component of the spliceosome, responsible for recognizing and binding to the 5' splice site (5'ss) of pre-mRNA via its conserved 9-nucleotide 5' tail.⁵ The 165-nucleotide U1snRNA adopts a stable secondary structure and forms a complex with U1-specific proteins to initiate spliceosomal assembly. Modified versions of U1 snRNA, known as exon-specific U1 snRNAs (ExSpeU1s), are engineered to contain tailored 5' tails that bind to intronic sequences downstream of canonical 5'ss.¹⁹ These modifications enable ExSpeU1s to promote exon inclusion and effectively rescue aberrant exon skipping in various splicing disorders,^{19,23–26} including cystic fibrosis,²⁷ hemophilia,^{24,26,28,29} and spinal muscular atrophy.^{23,30} ExSpeU1-mediated rescue has several advantages and promising therapeutic potential. First, by specifically targeting mRNA, gene expression can be efficiently restored while maintaining endogenous transcriptional regulation, limiting the toxic effects of overexpression that are typical of gene supplementation therapies. Second, the compact size of the ExSpeU1 cassette enables splicing correction of genes whose full-length transcripts exceed the packaging capacity of viral vectors, as is the case for *ELP1*. Recent findings indicate that systemic administration of ExSpeU1 was able to correct *ELP1* splicing and rescue survival and gait in the phenotypic FD mice with overall minimal global changes in *Elp1* splicing.²² To evaluate the therapeutic efficacy of ExSpeU1 in specifically correcting the *ELP1* splicing defect in the retina and preventing RGC death in FD, we optimized a novel adeno-associated virus serotype 2 (AAV2)-mediated intravitreal delivery approach of FD-ExSpeU1 and evaluated the rescue of RGC survival and function.

Intravitreal administration of self-complementary AAV2-ExSpeU1 (scAAV2-FD-ExSpeU1) in a phenotypic mouse model of FD rescues RGC survival. More importantly, FD-ExSpeU1 delivery to the retina significantly improved visual function in FD phenotypic mice. The results of this study provide critical proof-of-concept data for the development of a retina-specific splicing modulatory therapy for FD.

RESULTS

Design and development of scAAV2-FD-ExSpeU1-eGFP for retinal delivery

Mutations in the 5'ss can compromise U1 snRNA binding, impair spliceosome assembly, and consequently lead to exon skipping, as observed in FD. ExSpeU1s are ~86-nucleotide modified U1 snRNAs that are designed to bind intronic sequences via a modified 5' tail. Encoded by a compact ~700 bp region, they are well-suited for *in vivo* delivery using AAV. By targeting the 5'ss downstream of specific exons, ExSpeU1s can correct aberrant splicing in various cellular and mouse models.^{23–27,30,31} Additionally, they can promote the inclusion of exons that are normally alternatively spliced.³² To restore defective splicing, ExSpeU1s have been created with sequence changes that permit targeted binding to intronic sequences downstream of the mutant 5' ss.^{23,27,30,33} Our previous work has explored the use of the minigene system to screen ExSpeU1s that can efficiently correct the *ELP1* exon 20 splicing of C.2204 + 6 T>C mutation. Using this assay, we have identified Ik10 as the most effective ExSpeU1 snRNA species that can correct exon 20 splicing both *in vitro* and *in vivo*.¹⁹ Additionally, we have also shown that AAV9-mediated delivery of ExSpeU1 via intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) routes leads to significant improvement of gait ataxia, renal, and cardiac function in FD mice.²² Given the recent success in retinal gene therapy and the impact of vision loss in FD patients, we asked whether local delivery of FD-specific ExSpeU1 can correct *ELP1* splicing in the retina and prevent RGC loss in the FD phenotypic mouse *TgFD9;Elp1^{Δ20/flox}*.^{15,16} This mouse model was generated by our group in 2016¹⁶ by introducing the human *ELP1* transgene, *TgFD9*, into a hypomorphic mouse that expresses very low levels of endogenous *Elp1* (*Elp1^{Δ20/flox}*).^{16,34,35} The *TgFD9* transgene consists of a human bacterial artificial chromosome/clone containing the entire *ELP1* gene, including its native promoter and regulatory regions, into which we inserted the major FD splicing mutation (c.2204 + 6T>C).³⁵ The endogenous mouse alleles include one hypomorphic *Elp1^{flox}* allele, which produces reduced levels of full-length *Elp1* transcript, and one *Elp1^{Δ20}* allele, in which exon 20 is deleted and thus does not yield any full-length *Elp1* mRNA. To date, this is the most robust and physiologically relevant FD model available in the field, as it faithfully recapitulates multiple phenotypic manifestations of the human disease, including the retinal phenotype, and reproduces the same tissue-specific *ELP1* exon 20 mis-splicing observed in patients.

To develop a novel ExSpeU1-based approach capable of efficiently and specifically correcting *ELP1* splicing defects in the retina, we have designed a recombinant self-complementary adeno-associated viral (AAV) plasmid vector, (scAAV-FD-ExSpeU1-eGFP),

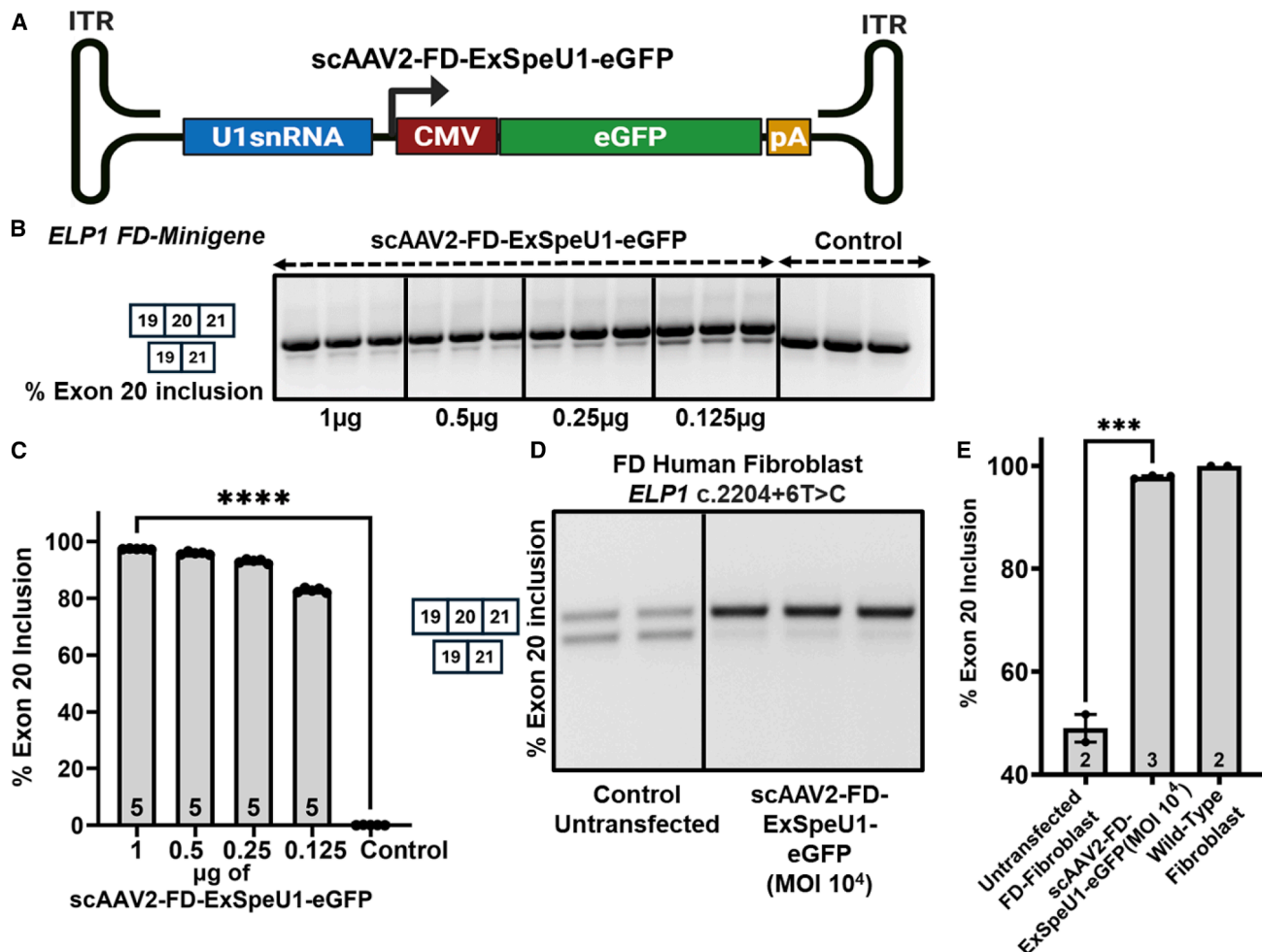


Figure 1. Design and *in vitro* testing of AAV2 vector expressing FD-ExSpeU1

(A) Schematic of the AAV vector expressing FD-ExSpeU1 and eGFP. FD-ExSpeU1 gene sequence, along with eGFP under the control of cytomegalovirus (CMV) promoter and bovine growth hormone (BGH polyadenylation A) (pA) sequences, was cloned between two inverted terminal repeat sequences (ITRs). (B) Representative gel picture showing splicing correction of FD minigene in HEK293T cells with increasing concentration of scAAV2-FD-ExSpeU1-eGFP vector ($n = 5$) (see also Figure S1). (C) Bar graph indicating the quantification of exon 20 splicing correction in FD minigene assay (**** $p < 0.0001$). (D) Representative gel picture showing complete correction of *ELP1* splicing in FD patient fibroblasts (*ELP1*c.2204 + 6T>C) transfected with scAAV2-FD-ExSpeU1-eGFP (see also Figure S2). (E) Bar graph indicating the quantification of exon 20 splicing correction in FD Fibroblast (*ELP1*c.2204 + 6T>C) transfected with scAAV2-FD-ExSpeU1-eGFP ($n = 3$). Statistical analysis was performed using ordinary ANOVA to compare the differences between the groups. Adjusted p values are shown as **** $p < 0.0001$, *** $p < 0.001$.

consisting of a sequence encoding ExSpeU1 (U1snRNA) and an eGFP reporter gene under the control cytomegalovirus (CMV) promoter. These two genes are flanked by two inverted terminal repeat (ITR) sequences to generate a plasmid vector compatible with AAV production. (Figure 1A). We chose to express eGFP along with FD-ExSpeU1 to study the retina-specific expression of FD-ExSpeU1. The efficacy of FD-ExSpeU1 in promoting inclusion of *ELP1* exon 20 was first evaluated using our well-established FD minigene splicing assay.^{36,37} A minigene containing *ELP1* exons 19, 20, and 21 and introns 19 and 20, along with the FD splice site mutation, was transfected into HEK293T cells with the plasmid scAAV-FD-ExSpeU1-eGFP. Transfection of increasing concentrations of scAAV-FD-ExSpeU1-eGFP corrected *ELP1* splicing in a dose-dependent

manner (Figures 1B, 1C, and S1) ($n = 5$). To deliver FD-ExSpeU1 to the retina and, more specifically, to the RGCs, the retinal neurons that are affected in FD, we have produced a self-complementary AAV2 expressing ExSpeU1 (scAAV2-FD-ExSpeU1-eGFP). AAV2 capsid was used to deliver ExSpeU1 based on its reported tropism for mouse RGCs and its use and tolerance in the human eye.^{38,39} To initially test the efficacy of scAAV2-FD-ExSpeU1-eGFP in correcting *ELP1* splicing *in vitro*, FD fibroblasts obtained from patients (containing c.2204 + 6T>C mutation in the *ELP1* gene) were transfected with scAAV2-FD-ExSpeU1-eGFP for 48 h. Real-time PCR analysis to detect the *ELP1* transcripts indicated the complete correction of the splicing defect in FD fibroblasts compared to the control untransduced fibroblasts (Figures 1D, 1E, and S2) ($n = 3$).

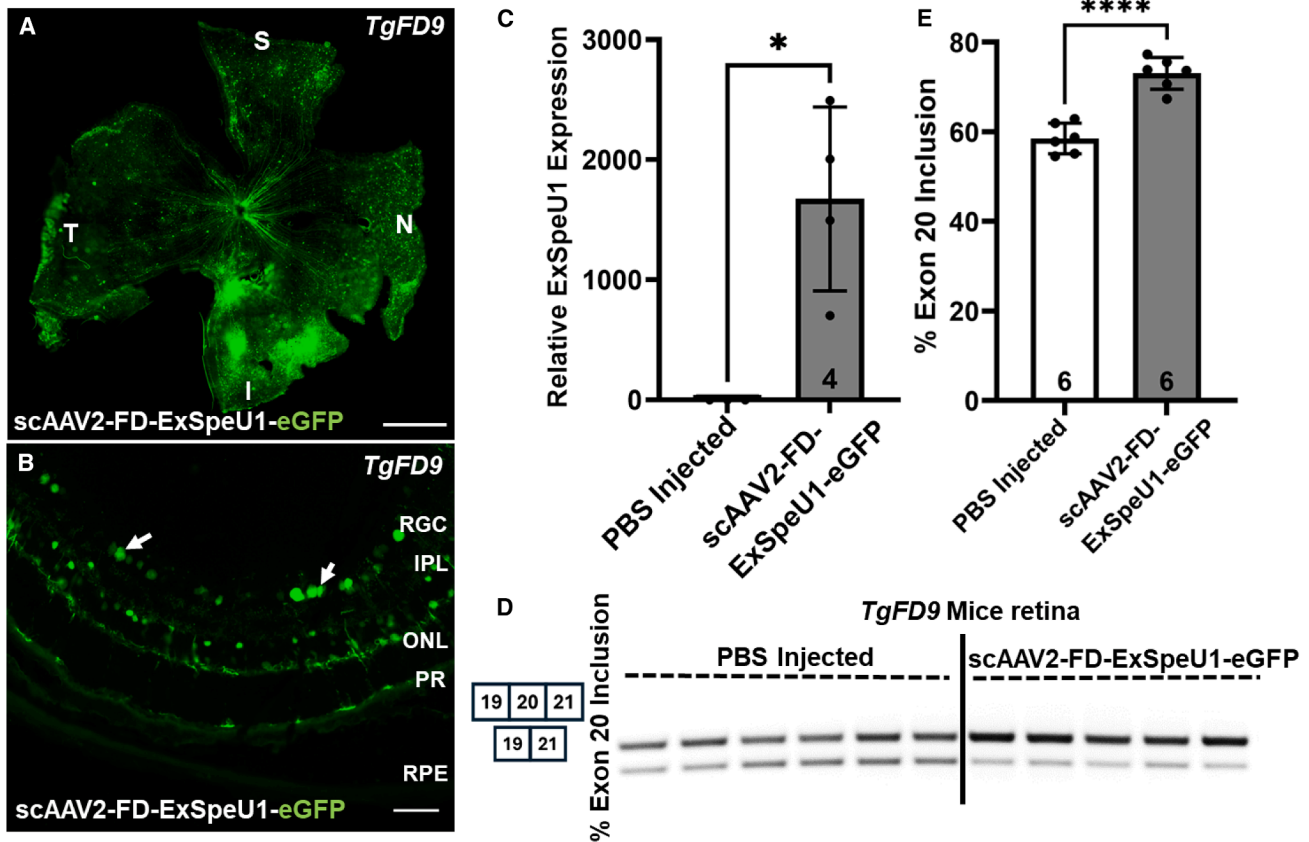


Figure 2. scAAV2-FD-ExSpeU1-eGFP corrects *ELP1* splicing defect in the retina

(A) Representative image showing retinal flat mount from *TgFD9* mice intravitreally injected with scAAV2-FD-ExSpeU1-eGFP. Staining was performed using an anti-GFP antibody. (B) Image of retinal cross-section obtained from *TgFD9* mice stained with anti-GFP antibody. The arrow indicates GFP+ve cells in the RGC layer of the retina. (C) Bar graph representing the relative expression of FD-ExSpeU1 in the retina of FD phenotypic mice injected with scAAV2-FD-ExSpeU1-eGFP compared to PBS-injected control 6 months after injection ($n = 4$; $p < 0.053$). (D) Representative splicing analysis of human *ELP1* transcripts (both the PCR products from the gel were confirmed by Sanger sequencing) (see also Figure S3). (E) Percent of exon 20 inclusion from scAAV2-FD-ExSpeU1-eGFP ($n = 6$, light gray). *** $p < 0.001$, two-tailed unpaired Student's *t* test with false discovery rate (FDR) correction.

Intravitreal delivery of scAAV2-FD-ExSpeU1-eGFP corrects *ELP1* splicing in the retina

We further aimed to establish the most effective ExSpeU1 delivery system to correct the *ELP1* splicing defect in the retina. We initially employed the asymptomatic *TgFD9* transgenic mice harboring the human *ELP1* transgene with the c.2204 + 6T>C FD mutation. These mice are phenotypically normal as they express normal levels of endogenous mouse *Elp1*. This is the best model to assess the effect of our ExSpeU1-based approach on *ELP1* splicing. We chose to utilize *TgFD9* mice for this analysis, as phenotypic FD mice are more difficult to generate due to their low survival rate.¹⁶ We administered intravitreal injections of scAAV2-FD-ExSpeU1-eGFP into *TgFD9* mice at postnatal day 21 (P21), delivering 1×10^{10} viral genomes (VGs) in a volume of 1 μ L per eye ($n = 4$). This dose was selected based on prior studies demonstrating its efficacy and safety as an optimal range for intravitreal delivery in murine models.^{40,41} Notably, similar dosing strategies have been employed in clinical trials targeting LHON, further supporting its translational relevance.⁴¹

We then evaluated the transduction efficacy of scAAV2-FD-ExSpeU1-eGFP 30 days post-injection by staining retinal flat mounts with a GFP-specific antibody. Intravitreal administration of scAAV2-FD-ExSpeU1-eGFP resulted in the expression of eGFP throughout the retina, indicating significant transduction efficiency (Figure 2A). The expression of eGFP was predominantly observed in RGCs, as evidenced by the GFP staining of the retinal cross sections obtained from the eyes injected with scAAV2-FD-ExSpeU1-eGFP (Figure 2B). In addition, to specifically evaluate the expression of ExSpeU1 in the retinas injected with scAAV2-FD-ExSpeU1-eGFP in FD phenotypic mice, quantitative PCR (qPCR) analysis was performed. A significant expression of ExSpeU1 was detected in injected retinas of FD mice compared to 1XPBS-injected control retinas (Figure 2C) ($n = 6$; $p < 0.053$). We further analyzed the correction of *ELP1* splicing in the retinas injected with scAAV2-FD-ExSpeU1-eGFP, which showed significant correction of the splicing compared to control ($n = 4$; $p < 0.001$) (Figures 2D, 2E, and S3). These results indicate efficient retinal targeting of the

scAAV2-FD-ExSpeU1-eGFP vector and significant correction of *ELP1* splicing with intravitreal delivery of ExSpeU1.

scAAV2-FD-ExSpeU1-eGFP treatment rescues RNFL loss in FD mice

We have previously shown that FD mice exhibit significant RNFL and GCIPL loss starting at 3 months, with further reduction at 6 months.¹⁵ The progressive thinning of these retinal layers perfectly recapitulates the disease phenotype observed in FD patients.¹⁵ To test whether delivery of scAAV2-FD-ExSpeU1-eGFP can rescue RNFL and GCIPL thinning, we performed intravitreal injections of scAAV2-FD-ExSpeU1-eGFP at a total dose of 1×10^{10} VGs at P30 in the phenotypic FD mice. One eye in FD mice is injected with scAAV2-FD-ExSpeU1-eGFP, and the contralateral eye is injected with 1XPBS (sham). To evaluate the rescue of RNFL and GCIPL thickness *in vivo*, we performed a spectral domain-optical coherence tomography (SD-OCT) analysis. We measured RNFL thickness in the superior, inferior, nasal, and temporal hemispheres of the retinas at 6 months of age in injected mice (Figure 3A). We observed a significant reduction of the RNFL in each region of the PBS-injected retinas (sham) compared to control littermates (control, $n = 17$; PBS-injected, $n = 8$) (central temporal, $p < 0.0017$; central nasal, $p < 0.0021$; peripheral temporal, $p < 0.0001$; and peripheral nasal, $p < 0.0001$). Interestingly, retinas injected with scAAV2-FD-ExSpeU1-eGFP showed significant improvement in the RNFL thickness compared to sham (scAAV2-FD-ExSpeU1-eGFP, $n = 9$) (central temporal, $p < 0.0107$; central nasal, $p < 0.0337$; peripheral temporal, $p < 0.0030$; and peripheral nasal, $p < 0.0253$) (Figure 3B). Similarly, GCIPL thickness was significantly reduced in FD mice compared to control reconfirming our previous findings in FD mice¹⁵ (control, $n = 16$; PBS-injected, $n = 7$) (central temporal, $p < 0.0038$; central nasal, $p < 0.0010$; peripheral temporal, $p < 0.028$; and peripheral nasal, $p < 0.0010$). In addition, GCIPL thickness was improved in retinas injected with scAAV2-FD-ExSpeU1-eGFP in all retinal regions compared to saline-injected retinas (scAAV2-FD-ExSpeU1-eGFP, $n = 7$) (central temporal, $p < 0.0104$; central nasal, $p < 0.0203$; peripheral temporal, $p < 0.001$; and peripheral nasal, $p < 0.0028$) (Figure 3C). These results indicate that delivery of scAAV2-FD-ExSpeU1-eGFP was able to improve RNFL and GCIPL thickness, supporting the efficacy of our approach in rescuing retinal nerve fiber in FD mice.

scAAV2-FD-ExSpeU1-eGFP treatment improves visual function in FD mice

To this end, our results demonstrate that intravitreal delivery of scAAV2-FD-ExSpeU1-eGFP significantly improves *ELP1* splicing in the retina, increases RNFL and GCIPL thickness, in FD phenotypic mice. To confirm that rescue of RGC translates to a functional improvement, we conducted a comprehensive assessment of retinal physiology in treated mice. Specifically, we used pattern electroretinography (PERG), a non-invasive, objective method that selectively measures RGC activity,^{42,43} to quantify RGC function in FD mice injected with scAAV2-FD-ExSpeU1-eGFP. Since we previously observed significant RGC loss by 6 months of age in FD

phenotypic mice,¹⁸ we assessed treated and control animals at this time point.

Our findings revealed a marked reduction in PERG amplitude (Figures 4A and 4B) in FD phenotypic mice ($n = 6$) compared to controls ($n = 11$; $p < 0.0062$), indicating significant RGC dysfunction. Notably, eyes treated with scAAV2-FD-ExSpeU1-eGFP exhibited significant restoration of PERG amplitude compared to PBS-injected controls ($n = 6$; $p < 0.0085$), demonstrating that ExSpeU1-mediated correction of *ELP1* splicing not only preserves RGC survival but also restores their functional output (Figures 4A and 4B). These results establish a critical proof-of-concept: molecular rescue of RGCs through splicing correction can lead to functional visual improvement in a mammalian model of FD.

To further evaluate the rescue of visual acuity as an indirect measure of “functional vision” in FD mice injected with scAAV2-FD-ExSpeU1-eGFP and control littermates, we employed an optomotor reflex-based spatial frequency threshold test known as the optomotor response (OMR) assay (Figures 4C and 4D). The results from OMR analysis showed a significant reduction in optomotor reflex in FD mice compared to the control mice ($n = 6$; $p < 0.0075$). Importantly, a significant improvement in spatial frequency is observed in eyes injected with scAAV2-FD-ExSpeU1-eGFP compared to PBS-injected eyes ($n = 8$; $p < 0.0399$). (Figure 4D). Taken together, these findings demonstrate that FD phenotypic mice exhibit significant visual dysfunction by 6 months of age, and with targeted *ELP1* splicing modulation via FD-ExSpeU1 delivery, effectively restore visual function *in vivo*. Importantly, this is the first demonstration that anatomical rescue of RGCs leads to a measurable improvement in visual function, providing direct evidence that *ELP1* splicing correction can rescue and restore RGC functionality.

Delivery of scAAV2-FD-ExSpeU1-eGFP to the retina ameliorates RGC degeneration in FD mice

Progressive death of RGCs postnatally has been shown in well-characterized mouse models of FD.^{44,45} Our previous study to characterize retinal phenotype in the FD mouse model indicated a significant loss of RGCs at 6 months.^{15,17} To validate the OCT findings and functional rescue with histological evidence, mice were sacrificed, and retinal flat mounts were prepared from scAAV2-FD-ExSpeU1-eGFP-treated and PBS-injected FD eyes. RGCs were counted across the superior, inferior, nasal, and temporal regions using RNA-binding protein with multiple splicing (RBPMS) staining. (Figure 5A). Consistent with our previous observations, the number of RBPMS +ve cells is significantly lower in PBS-treated retinas in all the retinal regions compared to retinas from control littermates ($n = 5$; superior, $p < 0.0003$; inferior, $p < 0.0329$; temporal, $p < 0.0471$; and nasal, $p < 0.0172$). Importantly, significant rescue of RGC loss was observed in the retinas treated with scAAV2-FD-ExSpeU1-eGFP ($n = 7$) in the temporal, nasal, superior, and inferior regions of the retina compared to the saline-injected retinas ($n = 5$; superior, $p < 0.0449$; inferior, $p < 0.0473$; temporal, $p < 0.0284$; and

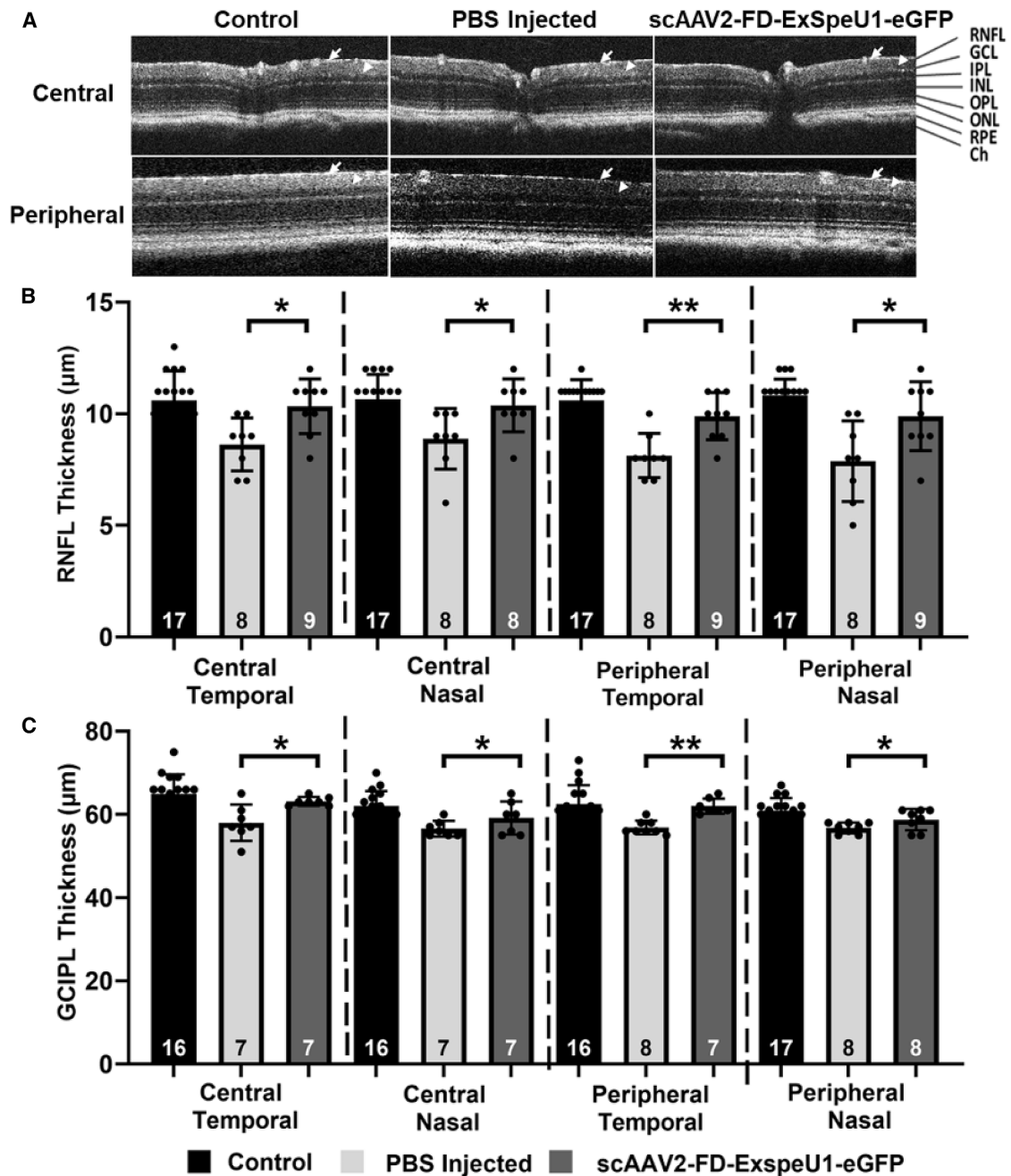


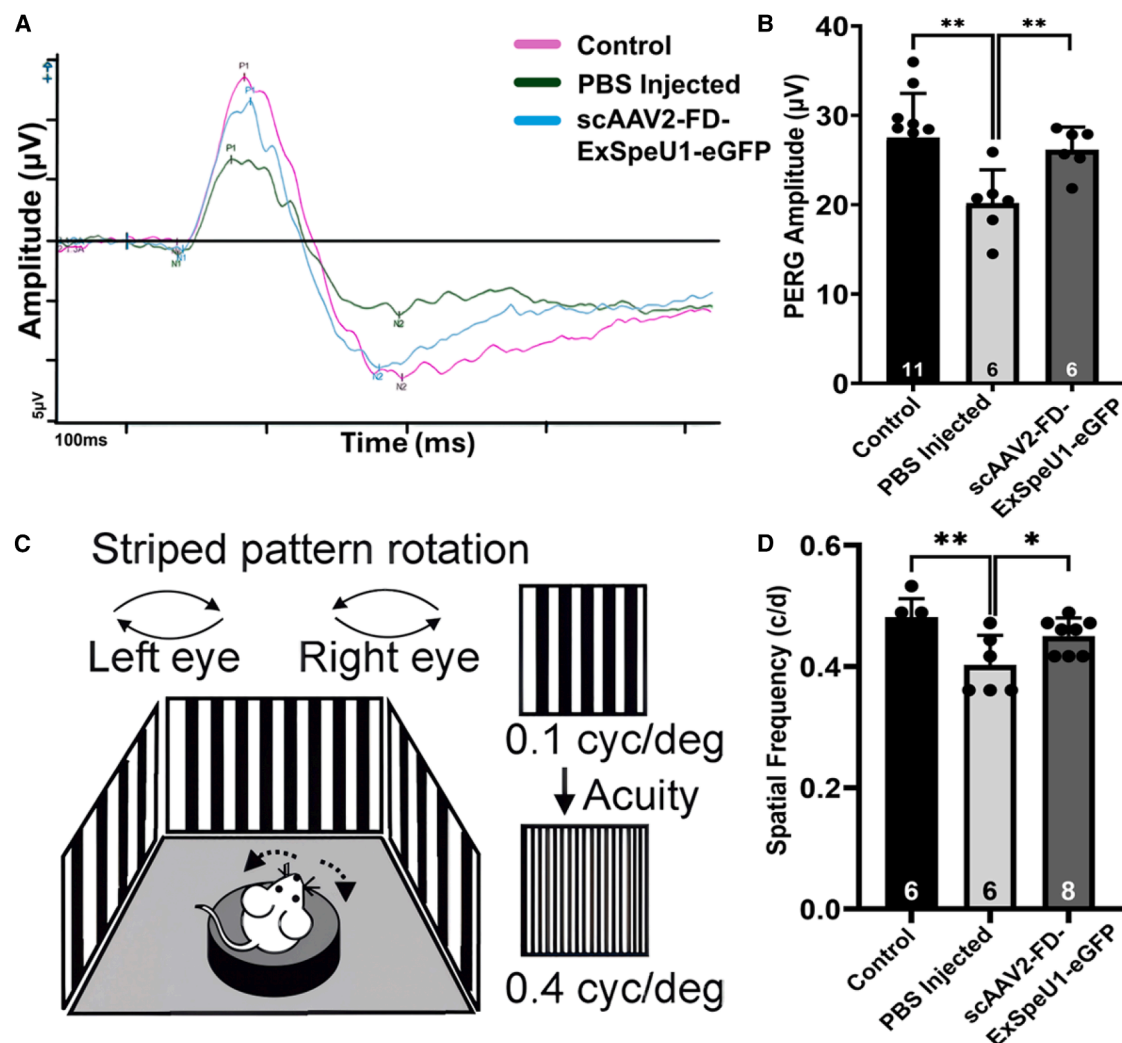
Figure 3. Intravitreal delivery of *scAAV2-FD-ExSpeU1* improves RNFL and GCIPL thickness in FD mice

(A) Representative image of SD-OCT analysis in FD mice intravitreally injected with *scAAV2-FD-ExSpeU1-eGFP* 6 months post-injection. One eye of the FD mice is injected with AAV, and the contralateral eye is injected with 1XPBS. (B) Measurement of RNFL thickness in both temporal and nasal hemispheres in central and peripheral regions of the retina in control ($n = 17$) and *scAAV2-FD-ExSpeU1-eGFP*-injected eyes ($n = 9$) and PBS-injected in FD mice ($n = 8$). (C) Bar graph representing improvement in GCIPL thickness in *scAAV2-FD-ExSpeU1-eGFP*-injected FD eyes ($n = 7$) compared to PBS-injected eyes ($n = 7$) (control, $n = 16$). The adjusted p values are displayed. * $p < 0.05$, ** $p < 0.01$, two-tailed unpaired Student's t test with FDR correction. Data are shown as average $\pm$ SEM; each data point represents an individual retina.

nasal, $p < 0.0026$) (Figures 5B and S4). Together, these results demonstrate that intravitreal delivery of ExSpeU1 can halt the progression of RGC loss observed in FD mice.

DISCUSSION

FD is a complex neurologic disorder characterized by combined neurodevelopmental defects and progressive neurodegeneration. Optic



(A) Representative superimposed PERG wave forms of control (purple), PBS injected (green) (left) and scAAV2-FD-ExSpeU1-eGFP injected (blue) (right) eyes. (B) Bar graph indicating mean PERG amplitudes (μV) of recordings from each FD mouse eye 6 months after intravitreal injection of scAAV2-FD-ExSpeU1-eGFP (right eye) ($n = 6$; $p < 0.0062$) or PBS-injected ($n = 6$) (left eye) and control mice ($n = 11$). Significant improvement in PERG amplitude is observed in scAAV2-FD-ExSpeU1-eGFP injected eyes compared to PBS-injected eyes ($n = 6$; $p < 0.0085$). (C) A simplified illustration of the OMR assay. High-contrast visual stimulation was applied to track reflexive head movements in response to the rotation of a moving stripe pattern with increased spatial frequency measured as cycles per degree (cycles/degree). (D) Bar graph representing the OMR of each mouse eye measured as spatial frequency (cycles/degree) in FD mice 6 months after intravitreal injection of scAAV2-FD-ExSpeU1-eGFP (right) ($n = 8$; $p < 0.0075$) and PBS-injected (left) ($n = 6$) eyes, and control mice ($n = 6$; $p < 0.0399$). The data were analyzed with a two-tailed unpaired Student's t test with FDR correction. Adjusted p values are shown: * $p < 0.05$; ** $p < 0.01$.

neuropathy in FD is a hallmark of the disease, and it resembles well-characterized optic neuropathies such as LHON (Leber hereditary optic neuropathy) and DOA (dominant optic atrophy).^{8–10} Despite progress being made in developing therapies, there is currently no effective treatment for optic neuropathy in FD. All patients suffer from progressive visual decline and will eventually become legally blind by their third decade of life.^{8–10} The eye is an easily accessible organ amenable to localized therapeutic intervention, making it ideal for testing the therapeutic efficacy of various treatments using functional, quantifiable parameters. Recent clinical success in mitigating

retinal cell loss in retinal dystrophy patients has demonstrated that gene supplementation strategies that introduce a normal copy of a mutant gene can dramatically improve visual function, which led to the first FDA-approved gene therapy drug, Luxturna.⁴⁶ These successful endeavors in retinal gene therapy, combined with the recent demonstration that systemic treatment with an SMC rescues the FD phenotype in a mouse model, have provided the foundation for our development of a retina-specific splicing modulatory therapy using ExSpeU1 to provide hope that the drastic visual loss in FD patients can be rescued.

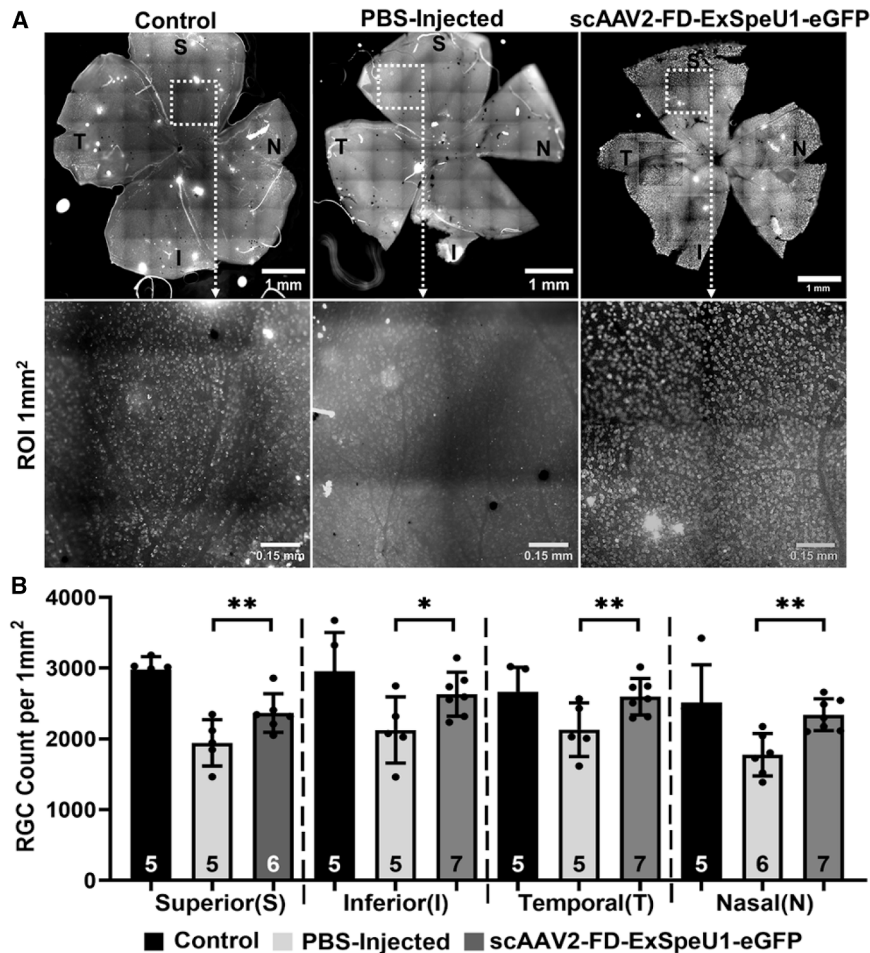


Figure 5. scAAV2-FD-ExSpeU1-eGFP ameliorates RGC degeneration in FD mice

(A) Representative flat-mount images of retinas from control mice, PBS-injected FD mice (Sham), and scAAV2-FD-ExSpeU1-eGFP-injected FD mice at 6 months of age. Scale bars: 1 mm. RBPMS+ve RGCs were quantified in the superior, inferior, nasal, and temporal quadrants at 1 mm from the optic nerve head (ONH). The lower panel shows higher magnification images highlighting RGC staining in each group (Scale bars: 1 mm). (B) Quantification of RBPMS+ve cells in control ($n = 5$), PBS-injected FD ($n = 5$), and scAAV2-FD-ExSpeU1-eGFP-injected FD ($n = 7$) mice (superior [S]; $p < 0.0003$, inferior [I]; $p < 0.0329$, temporal [T]; $p < 0.0471$ and nasal [N]; $p < 0.0172$). Data are presented as mean $\pm$ SEM, with each data point representing an individual retina. Statistical analysis was performed using a two-tailed unpaired Student's t test with FDR correction. Adjusted p values are shown: * $p < 0.05$; ** $p < 0.01$.

Previous studies have shown that RGC loss in FD can be mitigated through splicing modulatory therapies or genetic supplementation with full-length *ELP1* delivered to the retina.^{17,18,47} However, until now, it remained unknown whether preventing RGC loss also translates into functional recovery of these neurons. Splicing modulation or *ELP1* gene supplementation can reduce RGC loss in FD, but it has been unclear whether this preservation restores function. We provide the first direct evidence that *ELP1* splicing modulation not only sus-

tains RGC survival but also restores measurable function, establishing a robust endpoint for therapeutic evaluation. Unlike the well-documented structure-function correlations in glaucoma, such relationships have not been shown in monogenic inherited optic neuropathies like FD.^{51,52}

Toxicity induced by AAV-mediated delivery has been one of the significant safety concerns in ocular gene therapy clinical and preclinical trials.^{53–55} This is mainly attributed to the accumulation of over-expressed protein in the target tissues and higher doses of AAV, leading to toxic immunologic responses.⁵⁶ Since FD-ExSpeU1 is a small non-coding RNA specifically targeting the mutated splice site, it does not necessarily promote changes in gene expression or alternative splicing.^{19,22} Therefore, we expect that ectopic expression of FD-ExSpeU1 will not result in the induction of a significant cellular stress response. Previous studies evaluating ExSpeU1 in dorsal root ganglia (DRG) and spinal cord tissues have shown specific splice correction with minimal off-target splicing perturbations,^{19,22–25} suggesting a low frequency of unintended transcriptome-wide effects. However, future work will include direct assessment of splicing specificity in retinal tissue to fully establish safety

Progressive loss of RGCs and optic nerve degeneration are characteristic features of optic neuropathy in FD. In this study, we showed that intravitreal delivery of FD-ExSpeU1 can correct *ELP1* splicing defects in the retina and rescue the retinal phenotype in FD. Significant rescue of RNFL and GCIPL thickness in the retinas injected with FD-ExSpeU1 at P30 indicates that delivery of FD-ExSpeU1 at postnatal stages is sufficient to prevent RGC death. Our findings are consistent with earlier studies showing that RGC death can be rescued by oral administration of the splicing modulator compound PTC680, even when the treatment started at 3 months of age in a mouse model of FD.⁴⁷ This rescue is attributable to the postnatal nature of RGC death in FD. Together, these results support the idea that there is a broad therapeutic window for treating vision loss in FD patients. In this study, sustained rescue of RGC structure and function was observed up to 6 months post-injection, which is consistent with the well-documented long-term persistence of AAV-mediated transgene expression in retinal and systemic tissues. Previous preclinical and clinical studies have demonstrated that AAV vectors can maintain stable therapeutic expression for years,^{48–50} supporting the likelihood that the ExSpeU1-mediated correction will extend beyond the time points evaluated in this study.

in the ocular context. In addition, the scAAV2 serotype used in this study predominantly targets the RGC population, which is known to be affected in the FD retina.^{38,39} The AAV dose used in our experiments was 1×10^{10} VG total, which is efficient in the correction of splicing defects in the retina. This *in vivo* dose was informed by *in vitro* studies, where we observed that an multiplicity of infection (MOI) of 1,000 vg/cell achieved a near complete *ELP1* splicing correction in FD fibroblasts.^{41,57} Given approximately 1 million RGCs in the mouse retina,⁵⁸ this dose corresponds to a theoretical MOI of $\sim 10,000$ vg/cell. Adjusting for $\sim 30\%$ transduction efficiency with intravitreal AAV2 delivery,⁴⁰ the effective MOI ($\sim 3,000$ – $3,500$ vg/cell) remains within the range shown to be efficacious *in vitro*, supporting the translational relevance of our dosing strategy. In addition, this dose is comparable to the dosage used in the LHON gene therapy trial (2.4×10^{10} VG),⁴¹ which showed no serious safety concerns in a phase 1 clinical trial.⁵⁹ Even though our data indicate the efficacy of FD-ExSpeU1 in correcting *ELP1* splicing defect and preventing RGC degeneration in FD, we recognize that preclinical dose escalation and safety studies should be performed in the future for the translation of this technology to the clinic. Although intravitreal AAV2 administration is generally well-tolerated, innate and adaptive immune responses, including the development of neutralizing antibodies, have been reported in both preclinical and clinical settings.^{60,61} These considerations will be important for future translational development, particularly with respect to dose optimization and the feasibility of repeat administration. For the advancement of ExSpeU1 as a therapeutic strategy for FD, several translational considerations will be important moving forward. These include the scalability of vector manufacturing under GMP (good manufacturing practice) conditions, the durability of splice correction over several years, and the potential impact of pre-existing anti-AAV neutralizing antibodies on both efficacy and eligibility for repeat administration if required. Evaluation in larger animal species, such as NHPs or canine models, will also be required to refine intravitreal dosing parameters, assess biodistribution to off-target ocular tissues, and monitor inflammatory or immunological responses under clinically relevant conditions. Together, these studies will provide a rigorous, stepwise framework to support the safe and effective translation of FD-ExSpeU1 toward treatment of optic neuropathy in patients with FD. Previous studies have shown that systemic correction of *ELP1* splicing defects through administration of FD-ExSpeU1 via i.p. or i.c.v. rescues motor and proprioceptive functions in FD mice.²² Even though these results are promising, there are several challenges for systemic administration of FD-ExSpeU1 in FD patients. First, systemic administration of AAV in several clinical and preclinical studies has been shown to exhibit hepatotoxicity due to the accumulation of AAV in the liver.^{62–64} Accumulation of AAV in DRG and associated toxicity are also commonly seen with systemic administration of AAV.⁶⁵ Second, the requirement of high vector dosage for systemic administration and associated costs to produce GMP-grade vector pose a major challenge for an ultra-rare disease such as FD.⁶⁶ Third, rescue of RGC loss with systemic delivery of FD-ExSpeU1 has not been evaluated. Therefore, there is no clear indication whether systemic deliv-

ery of AAV9-FD-ExSpeU1 will be efficacious in rescuing RGC loss in FD. On the other hand, oral delivery of a kinetin-derived SMC, such as PTC258 and PTC680, has been shown to rescue RGC loss in FD mouse models.^{17,47} These compounds are currently undergoing toxicology studies to evaluate their safety for potential clinical use. However, no approved treatment currently exists for FD-associated vision loss. Unlike small-molecule splicing modulators and ASO, which require continuous systemic dosing to maintain efficacy, intravitreal AAV delivery of ExSpeU1 enables long-term, retina-specific splicing correction from a single administration, and the two approaches are not mutually exclusive and may be used in combination. With the recent success of retinal gene therapies, there is now a viable and accelerating path to clinical translation for AAV-mediated approaches such as FD-ExSpeU1. In conclusion, our study provides the first evidence that targeted splicing correction using a modified U1 snRNA delivered intravitreally can not only prevent RGC loss but also restore visual function in a mouse model of FD. This functional rescue, achieved through scAAV2-FD-ExSpeU1, is particularly compelling given the precision of the approach and the markedly reduced off-target effects.^{19,22} Together, these findings underscore the therapeutic potential of ExSpeU1 for treating FD-associated optic neuropathy and establish a strong rationale for its advancement toward clinical development.

MATERIALS AND METHODS

Animals

The methodology for the creation of the transgenic mouse line *TgFD9* carrying the human *ELP1* transgene with FD mutation c.2204 + 6T>C, and the mouse lines carrying *Elp1*^{fllox}, and *Elp1* ^{$\Delta 20$} alleles were previously published.^{16,34,35} The experimental mice used for this study, *F2:TgFD9; Elp1* ^{$\Delta 20$ /fllox} were generated by crossing the heterozygous mice for *TgFD9* transgene (*TgFD9*^{+/-}) and the homozygous mice for the *Elp1* floxed allele (*Elp1*^{fllox/fllox}) to generate F1 mice (*F1:TgFD9*^{+/-}; *Elp1*^{fllox/fllox}). Pups were genotyped to identify those carrying both the *TgFD9* transgene and the *Elp1*^{fllox} allele (*F1:TgFD9*^{+/-}; *Elp1*^{fllox/fllox}). These F1 animals were then subsequently crossed with the mouse line heterozygous for the *Elp1* ^{$\Delta 20$} allele (*Elp1* ^{$\Delta 20$ /+}) to generate the FD mouse (*F2:TgFD9; Elp1* ^{$\Delta 20$ /fllox}). The F2 mice are referred to as FD mice henceforth in the manuscript. Controls are littermates of the FD mice that carry the transgene but are phenotypically normal because they express the endogenous *Elp1* gene (*TgFD9; Elp1*^{+/+}, *TgFD9; Elp1*^{fllox/+}, or *TgFD9; Elp1* ^{$\Delta 20$ /+}). The expected Mendelian ratio of the FD mouse with this breeding scheme was 1 in 4 (25%). However, only 50%–60% of FD mice survive postnatally due to underlying systemic abnormalities caused by the reduction of *Elp1*⁶⁴ therefore, the actual ratio is 1:8 (12.5%).¹⁸ Control and FD mice have a mixed background, including C57BL/6J and C57BL/6N. All the mice enrolled in the study were negative for the spontaneous rd8 mutation, which causes retinal degeneration in the C57BL/6J strain.

The mice used for this study were housed in the animal facility at Massachusetts General Hospital (Boston, MA), provided with access to food and water ad libitum, and maintained on a 12-h light/dark

cycle, and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital, and were in accordance with NIH guidelines. For routine genotyping of progeny, genomic DNA was prepared from tail biopsies and PCR was carried out using the following primers forward, 5'-TGATTGACACAGACTCTGGCCA-3'; reverse, 5'-CTT TCACTCTGAAATTACAGGAAG-3' to discriminate the *mouse Elp1* alleles and the primers forward 5'-GCCATTGTACTGTTTGC GACT-3' reverse, 5'-TGAGTGTACAGATTCTTCTGC-3' to detect the human *TgFD9* transgene.

Production of scAAV2-FD-ExSpeU1-eGFP

AAV vector to produce AAV2 expressing FD-ExSpeU1 was generated by cloning ExSpeU1 (750bp) and eGFP (700bp) expression cassettes into ITR containing self-complementary AAV2 backbone plasmid. The eGFP cassette is under the control of the CMV promoter, which is located upstream. The ExSpeU1 is expressed under the control of the endogenous U1 snRNA promoter, which drives proper nuclear expression and assembly of U1-derived transcripts.²⁷ Briefly, AAV2 is produced from endo-free plasmid DNA in a triple transfection (ITR-flanked FD-ExSpeU1-eGFP, packaging, and adeno-helper plasmids) in HEK293T (ATCC, #CRL-3216) cells using polyethylenimine. After 3 days, the viral vector is isolated from the combined lysate and cell media via sequential high salt, benzonase (for non-AAV protected DNA digestion) treatment, lysate clearing via high-speed centrifugation, tangential flow filtration (for volume reduction and clearing), iodixanol gradient ultracentrifugation (for purification and separation of empty vs. full particles), and buffer exchange and formulation in PBS via molecular weight cutoff filtration. The preparation underwent titration via digital droplet PCR, and quality control for purity was assessed via SDS-PAGE.

Intravitreal injections

Experimental and control mice were anesthetized with an i.p. injection of a ketamine/xylazine mixture (70 mg/kg ketamine and 20 mg/kg xylazine). Pupils of the mice were dilated using 2.5% phenylephrine and 1% tropicamide. Approximately 0.5% proparacaine is used as a topical anesthetic during the procedure for 1–2 min. GenTeal gel was used to keep the eyes moist and avoid corneal dryness and opacities. The body temperature of the mouse was kept stable at 37°C throughout the procedure. Under the control of a stereo microscope (Discovery.V20, Zeiss), an incision was made into the sclera posterior of the superior limbus using a sterile, sharp 30-gauge (G) needle without touching the lens. A microliter Hamilton syringe attached to a 34G blunt needle was carefully inserted into the same incision, and 1 µL of scAAV2-FD-ExSpeU1-eGFP or 1XPBS (sterile phosphate-buffered saline) was injected slowly into the vitreous using manual pressure. The needle was kept inside the vitreous area for at least 1 min after the injection to avoid reflux of viral suspension. The right eye is injected with scAAV2-FD-ExSpeU1-eGFP, and the contralateral eye is injected with 1XPBS. After the completion of intravitreal AAV injections, triple antibiotic ointment (neomycin, bacitracin, and polymyxin B) was applied, and

mice were injected subcutaneously with the analgesic buprenorphine (0.1 mg/kg).

SD-OCT

To evaluate the retinal structure, we performed *in vivo* imaging of the retina using SD-OCT. The mice were anesthetized by a ketamine/xylazine mixture as described above. Pupils of the mice were dilated using 2.5% phenylephrine and 1% tropicamide. Approximately 0.5% of proparacaine is used as a topical anesthetic during the procedure. SD-OCT imaging was performed using a Leica EnvisuR2210. RNFL and GCIPL measurements were performed by 10-layer automated segmentation using the software DIVER (Leica). PBS-injected and scAAV2-FD-ExSpeU1-eGFP-injected eyes of control and FD mice at 6 months of age were analyzed. Each OCT image comprises 100 B-scans, with each B-scan consisting of 1,000 A-scans.

Retinal whole mounts

RGC staining and counting of retinal whole mounts were performed according to the method previously described by Ueki et al.^{44,45} Briefly, fixation of the eyes was performed at room temperature for 1 h in 4% paraformaldehyde (PFA), and the eyes were marked with a yellow tissue marking dye on the temporal surface. After fixation, retinas were isolated, with each temporal retina marked with a small cut. Relaxing cuts in the spherical retina were made on all four corners to flatten the retina. Non-specific binding was blocked by incubation with an animal-free blocker containing 0.5% Triton X-100 overnight at 4°C. RGC-specific anti-RBPMS antibody (1:500 dilution) (PhosphoSolutions #1830-RBPMS) was applied for 48 h at 4°C. Retinas were then washed 3 times with PBS and incubated with secondary antibody (1:1,000 dilution) (Thermo Fischer Scientific, Alexa Fluor 568 # A-11011) for 1 h at room temperature and mounted on slides. Images were acquired with a Leica DMi8 epifluorescent microscope and MetaMorph 4.2 acquisition software (Molecular Devices, San Jose, CA). Whole scans of complete flat-mount samples were obtained at 20× magnification via scan stage and autofocus. The total retinal area across the entire scan was approximately 14 mm². With ImageJ software, the number of RBPMS +ve cells was measured at 1 mm² in 1 mm from the optic nerve head (ONH) at the superior, inferior, temporal, and nasal hemispheres.⁴⁵ If a specific square area was damaged as a result of rips or folds in the retina, we counted the RGCs in an adjacent undamaged area. A customized analysis pipeline was developed in CellProfiler to objectively identify and count RBPMS+ve cells based on their characteristic size, intensity, and morphology. The pipeline included steps for image preprocessing, primary object (cell) identification, and intensity-based filtering to distinguish positive signals from background. Cell counts were performed within a defined 1 mm² region of interest positioned 1 mm from the ONH in each retinal quadrant (superior [S], inferior [I], nasal [N], and temporal [T]). The investigator conducting the analysis was blinded to the genotype.

Histology

Eyes were enucleated and fixed in 4% PFA overnight at 4°C. After a single 1XPBS wash, eyes were cryoprotected in 30% sucrose

overnight at 4°C and embedded in Tissue-Tek O.C.T. Compound (#4583 Sakura Finetek, Torrance, CA, USA) and cryo-sectioned into 15-µm-thin sections. Sections were fixed in dry ice-cold acetone for 15 min and washed with 1XPBS three times. Antigen retrieval was then performed by incubating the sections using the buffer containing 0.1 M Tris HCL pH 8, 50 mM EDTA pH 8.0, and 20 µg/mL Proteinase K for 10 min at room temperature. After washing two times in 1XPBS, retinal sections were blocked with an animal-free blocker (Vector Laboratories, Burlingame, CA, USA) containing 0.5% Triton X-100 for 1 h at room temperature. Primary antibody anti-GFP (1:1000 dilution) (green fluorescent protein) (Thermo Fisher Scientific #A10262, CA, USA) was applied and incubated at 4°C overnight in a moist chamber. Sections were washed three times with 1XPBS with 0.1% Triton X-100 and incubated with secondary antibodies (Invitrogen; Jackson Immuno Research, West Grove, PA, USA) for 1 h at room temperature. Sections were mounted with hard-set mounting media containing DAPI (#H-1500-10 Vector Laboratories), and fluorescent microscopy was performed. One eye from each mouse was included in this analysis.

Real-time qPCR

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect the expression of FD-ExSpeU1 using a real-time qPCR LightCycler 480 II (Roche). RNA was extracted from the retinas of either PBS-injected or scAAV2-FD-ExSpeU1-eGFP-injected mice using an RNA isolation kit (QIAGEN, #74534) and treated with DNase (QIAGEN, #79254). RNA yields were quantified using a NanoDrop ND-1000 spectrophotometer. RT-qPCR was performed for cDNA synthesis with 500 ng of starting total RNA in an 11.5 µL RT reaction. RT-qPCR was performed with iQ-SYBR Green Supermix (Bio-Rad Laboratories #1708880) using FD-ExSpeU1-specific primers (forward, 5'-ATAGCAAACAGTACAATGC-3' and reverse, 5'-CACTACCA CAAATTATGCA-3'). The expression levels of FD-ExSpeU1 were directly normalized with the housekeeping gene (*Gapdh*). RT-qPCR was carried out at the following temperatures for the indicated times: 95°C (3 min); 45 cycles of 95°C (15 s) followed by 60°C (30 s); and 98°C (0.11 s). Data were analyzed with the Thermo Fischer Scientific SDS qPCR analysis software.

Real-time PCR analysis of *ELP1* transcripts

Mice were euthanized, and eyes were enucleated in ice-cold 1XPBS. Retinas were dissected on ice under a microscope (Zeiss) and homogenized in ice-cold QIAzol Lysis buffer (QIAGEN, #79306) with QIAGEN tissue Lyser. Similarly, RNA was isolated with QIAzol Lysis Reagent from human fibroblasts treated with scAAV2-FD-ExSpeU1-eGFP, and total RNA was extracted using the standard phenol-chloroform extraction procedure. The concentration of the total RNA for each sample was determined with a Nanodrop ND-1000 spectrophotometer. cDNA was synthesized by reverse transcription with 0.5 µg of total RNA, Random Primers (Promega), and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. To perform RT-PCR and detect *ELP1* exon 20 inclusion, 100 ng of starting RNA equivalent cDNA was subjected to a PCR reaction with the GoTaq green master mix

(Promega, #M7122) and 30 amplification cycles (94°C for 30 s, 58°C for 30 s, and 72°C for 30 s). The human-specific forward and reverse primers spanning exon 19–21 of the *ELP1* 5'-CCTGAGCA GCAATCATGTG-3' and 5'-TACATGGTCTTCGTGACATC-3', were used for amplification of both wild-type and mutant isoforms of the human *ELP1* transcript. The PCR products were resolved on 1.5% agarose gels containing ethidium bromide for detection. The relative amounts of normal and mutant *ELP1* isoforms were quantified using ImageJ software, and the integrated density value for each band was determined and expressed as a percentage as described previously.¹⁶

Cell culture and minigene assay

HEK293T (ATCC, #CRL-3216) cells were cultured in DMEM (11995-065, DMEM, Gibco) supplemented with 10% fetal bovine serum ([FBS], 12306C-500 ML, Millipore Sigma) and 1% penicillin/streptomycin (30-009-CI, Corning). HEK293T cells (passage number 9) were transfected with FD minigene and scAAV2-FD-ExSpeU1-eGFP constructs and seeded in 6 wells. The following day, the media was changed to regular growth media. The total RNA was extracted using the standard chloroform extraction procedure 48 h after transfection to detect the *ELP1* transcripts. To specifically amplify the spliced *ELP1* transcript expressed by the FD minigene, T7 (5'-TAATACGACTCACTATAGGG) and BGHR (5'-TAGAAGG-CACAGTCGAGG) primers were used. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and visualized with the Alpha 2000 Imager Analyzer (Bio-Rad). Band intensities were quantified using ImageJ software. The expected sizes of the amplified products were approximately 480 base pairs for the mutant *ELP1* transcript and 550 base pairs for the wild-type *ELP1* transcript. Patient human fibroblasts carrying the c.2204 + 6T > C mutation in *ELP1* were obtained from Coriell Cell Repository (GM04663). Approximately 1×10^4 human FD fibroblast cells (passage number 20) were plated in 6-well plates and incubated at 37°C for 12 h. Cells were washed once with 1XPBS (room temperature) and then infected at 37°C either with phosphate-buffered saline (control) or with scAAV2-FD-ExSpeU1-eGFP vectors at a MOI of approximately 1,000 vgs/cell. At 4 h post infection, the virus was removed by washing the cells with DMEM/F12 media once, and the cells were supplemented with complete media. After 48 h of infection, cells were homogenized in ice-cold QIAzol Lysis buffer (QIAGEN, #79306), and the total RNA was processed according to the manufacturer's instructions. The isolated RNA was then subjected to real-time PCR as described earlier.

PERG

Mice were anesthetized and mounted on the Celeris PERG system (Diagnosys) platform and kept under dim red light throughout the procedure. A corneal electrode was placed on the right eye, and body temperature was stabilized at 37°C. The visual stimuli were presented at 98% contrast and constant mean luminance of 50 cd/m², spatial frequency of 0.05 cycle/degree, and temporal frequency of 1Hz. A total of 300 complete contrast reversals of PERG were repeated twice in each eye, and the 600 cycles were segmented,

averaged, and recorded. The averaged PERGs were analyzed to evaluate the peak-to-trough P1 to N2 amplitude as described previously.⁶⁷

OMR

To assess the visual function in the scAAV2-FD-ExSpeU1-eGFP-treated eye compared to the sham-treated eye, visual acuity in mice was assessed using an automated OMR machine (OptoDrum, STRIATECH GmbH). The OMR assay measures visual acuity by assessing the motor response of mice under photopic conditions to a virtual rotation of stripes of different widths. The OMR is evaluated via a spatial frequency threshold test. The equipment is composed of four computer monitors arranged in a quadrangle, which simultaneously display black and white striped patterns. The mouse is positioned on a pedestal at the center, directly below the camera. Tracking behavior was automatically detected and analyzed by OptoDrum software. This test includes an observer to make sure that all the mice's behavior is tracked throughout the experiment. The tracking is recorded only when the mice are sitting still, for a maximum of 20 s. Clockwise grating rotation was used to selectively stimulate the left eye (PBS-injected), whereas counterclockwise rotation stimulated the right eye (scAAV2-FD-ExSpeU1-eGFP treated).⁶⁸ Spatial frequency was increased using a staircase protocol while maintaining constant contrast (99.27%) and rotation speed (12°/s). The visual acuity threshold was defined as the highest spatial frequency that consistently elicited head-tracking behavior, requiring two positive responses and three negative responses at the next higher spatial frequency to confirm the threshold. The ExSpeU1-eGFP-injected right eye and PBS-injected contralateral control eye were tested separately.

Statistical analysis

Data are presented as mean $\pm$ SEM. Normality of all datasets was assessed using the Shapiro-Wilk test. For datasets that satisfied the assumption of normality ($p > 0.05$), statistical significance were evaluated using two-tailed unpaired Student's t tests or one-way ANOVA, as indicated in the figure legends. For datasets that did not meet the normality assumption, non-parametric tests were applied, including the Mann-Whitney U test. Multiple comparisons were corrected using false discovery rate adjustment. Statistical significance was defined as $p < 0.05$ ($p < 0.01$, $p < 0.001$, $p < 0.0001$). All statistical analyses were performed using GraphPad Prism software.

DATA AVAILABILITY

All data supporting the findings of this study are included in the published article and its supplemental information files. Additional raw datasets, including imaging files, qPCR data, and statistical analyses, are available from the corresponding author upon reasonable request. Plasmids encoding scAAV2-FD-ExSpeU1, *ELP1* FD minigene, and related AAV vector backbones can be provided to qualified researchers under a material transfer agreement with Massachusetts General Hospital.

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and vision research. experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital (#2006N000196). All efforts were made to minimize animal suffering and reduce the number of animals used. This work was supported by National Institutes of Health (NIH) grants R01 EY029544 (to S.A.S.), R01 EY036009 (to A.C.), R01NS124561 and R21EY037018 (to E.M.), Familial Dysautonomia Foundation (to A.C. and E.M.), and Knights Templar Eye Foundation (KTEF) (to A.C.).

AUTHOR CONTRIBUTIONS

E.M., S.A.S., and A.C. contributed equally to the conception and design of the study and are recognized as co-senior authors. A.C., E.M., and L.H.V. developed the methodology. A.C., K.K., E.G.K., M.C., S.K., D.K.-V., G.R., M.E.S., and J.B. performed the investigations. F.P., L.H.V., and S.A.S. provided resources. A.C., E.M., S.K., and K.K. curated the data, with analyses and visualizations. A.C., K.K., S.K., and E.M. drafted the manuscript. E.M., S.A.S., and A.C. supervised the work. S.A.S. secured the funding. All the authors reviewed and edited the final manuscript.

DECLARATION OF INTERESTS

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SUPPLEMENTAL INFORMATION

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