ABCA4 pre-mRNA Exon Editing *in vitro* and *in vivo*

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Background and summary

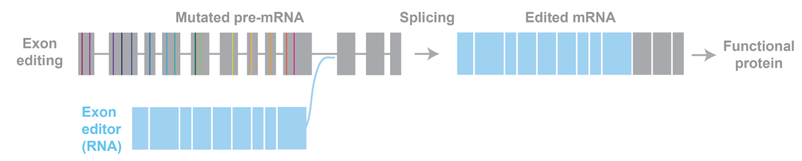
Ascidian Therapeutics has been pursuing RNA exon editing as a means to overcome historical barriers to progress in the gene therapy field. These barriers include, among others, the limited cargo capacity of AAV, the diverse spectrum of patient mutations in many diseases, and the difficulty of precisely controlling expression levels across diverse cell types. For example, gene replacement should in theory be able to cure Stargardt disease, which leads to inherited vision loss due to defects in the ABCA4 transporter. However, the ABCA4 coding sequence doesn't fit into AAV. Base editing should also be able to treat ABCA4 retinopathies such as Stargardt, but it is impractical because it would require a different clinically approved therapeutic for each of the hundreds of distinct pathogenic mutations.

To overcome these limitations, we are focused on pre-mRNA Exon Editing via spliceosome-mediated trans-splicing, which replaces a defective portion of a patient's pre-mRNA with an AAV-encoded synthetic RNA. Pre-mRNA Exon Editing overcomes the AAV capacity limit by making it productive to deliver portions of mRNAs. It also overcomes some of the difficulty of controlling expression levels, by restricting expression to a maximum that is determined by the expression of the pre-mRNA target in any given cell. Although attempts to apply trans-splicing to gene therapy were first described in 1999, it has for decades been hampered as a technology by its dependence on unknown rules of RNA structure, RNA splicing, and non-coding RNA function. Here we report pre-mRNA Exon Editing molecules that can efficiently replace a large portion of the 7 kb ABCA4 coding sequence, both in vitro and in the Non-Human Primate (NHP) retina. To achieve this performance, we developed a screening platform with the capacity to evaluate thousands of trans-splicing molecules in human cell culture and in the NHP retina. Using synthetic biology, next-generation sequencing, and bioinformatics, we screened thousands of molecules to identify the best ABCA4 pre-mRNA Exon Editors.

In evaluating individual pre-mRNA Exon Editors, we observed rescue of ABCA4 mRNA and ABCA4 protein expression in HEK293T cells engineered to express a defective ABCA4 pre-mRNA containing a premature stop codon. We also observed efficient editing of ABCA4 in the NHP eye, as measured from RNA or protein, one month after subretinal injections of AAV-encoded pre-mRNA Exon Editing constructs.

To our knowledge this study is the first to demonstrate pre-mRNA Exon Editing via RNA trans-splicing in NHPs.

Exon editing via pre-mRNA trans-splicing



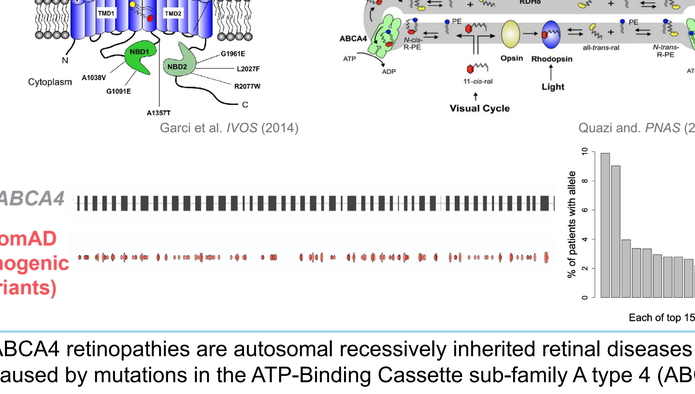
Previous pre-mRNA trans-splicing work had important limitations and challenges

Disease area attempts	Gene	Date(s) of report
Duchenne muscular dystrophy	*DMD*	2007, 2010
Dystrophic epidermolysis bullosa	*COL7A1, K14*	2007, 2013
Huntington's disease	*HTT*	2012, 2017
Cystic fibrosis	*CFTR*	2001, 2002, 2007
Spinal muscular atrophy	*SMN2*	2003, 2013, 2014
Dysferlinopathies / Titinopathies	*DYSF / TTN*	2005
Retinitis pigmentosa	*RHO*	2008
X-linked hyper-IgM	*CD40L*	2004

- Editors tested in artificial context
- Efficiency challenges
- Limited *in vivo* studies
- Lack of translational delivery strategies

Berger A, Maire S, Gaillard MC, Sahel JA, Hantraye P, Bemelmans AP. mRNA trans-splicing in gene therapy for genetic diseases. *Wiley Interdiscip Rev RNA*. 2016

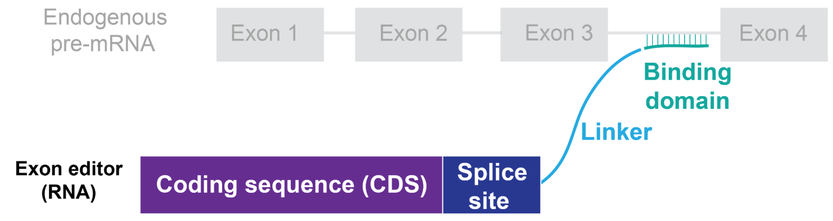
Advances in synthetic biology and next generation sequencing enable a high-throughput exon editing assay



Retinal disease gene ABCA4 is an excellent candidate for exon editing

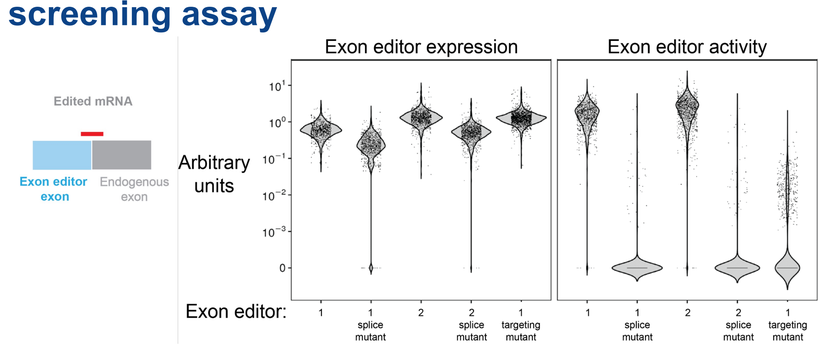
- ABCA4 retinopathies are autosomal recessively inherited retinal diseases caused by mutations in the ATP-Binding Cassette sub-family A type 4 (ABCA4) gene
- Loss of ABCA4 results in build up of fatty byproducts (lipofuscin) in the macula leading to cellular toxicity and a progressive loss of vision
- ABCA4 is too large (6.8 kb) for AAV-mediated gene replacement, and upwards of 900 unique mutations have been identified

Essential components of an exon editor



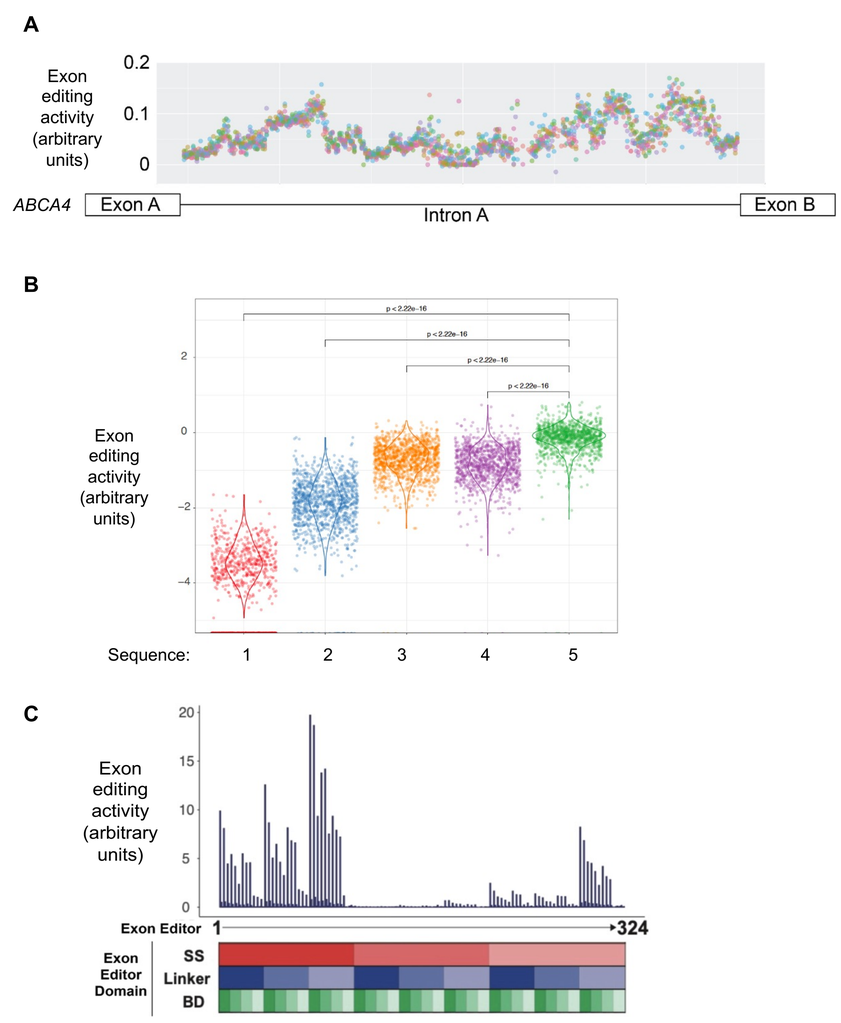
Exon editors are synthetic RNA-based molecules that contain a coding domain sequence, splice site, linker, binding domain, and other synthetic sequences to allow for specific interaction with endogenous pre-mRNA and subsequent replacement of target exons via trans-splicing. RNA exon editing molecules can be expressed from DNA that is delivered by an AAV (or non-viral) vector as a single AAV construct.

Establishing a high-throughput exon editing screening assay



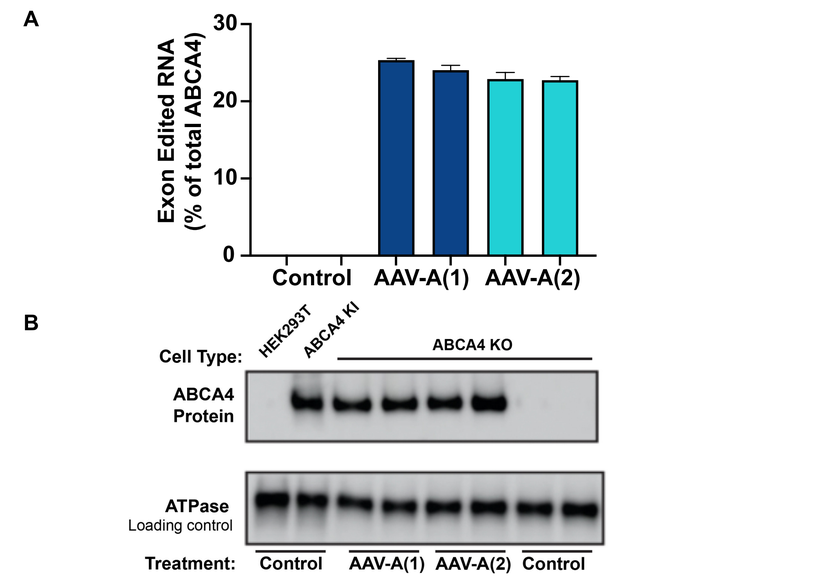
The expression levels of five exon editors are shown at left, with their activity shown at right. Expression and activity are measured by barcoded-RNA-seq. Within columns, each data point is a measurement for a unique barcode that represents an editor that is otherwise identical. Note the orders-of-magnitude lower activity of splice mutant and targeting mutant editors.

Screening and optimization of exon editor elements



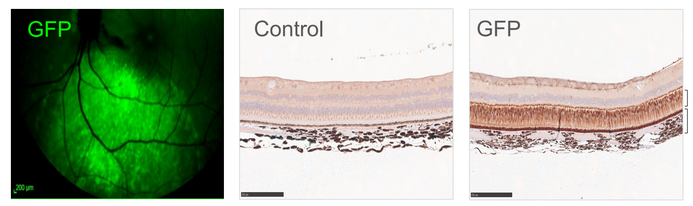
(A) Next generation sequence (NGS) based analysis exon editing activity of binding domain positions along an ABCA4 intron. Data from > 2,000 exon editors, tiled every 5 nucleotide along the intron is shown. (B) NGS-based analysis of exon editing activity using editors containing 5 unique splice sequences (colored respectively). Each individual data point represents a unique linker sequence tested. (C) NGS-based exon editing activity of 324 individual exon editors containing a combination of 3 splice sites (SS), 3 linker sequences, and 4 binding domains (BD). In all experiments exon editing activity is normalized to input of exon editors.

Rescue of ABCA4 protein expression *in vitro*



Protein rescue shown for two ABCA4 exon editors, AAV-A(1) and AAV-A(2) upon transduction into HEK293T-derived cells that were engineered to express a defective ABCA4 pre-mRNA containing a premature stop codon. The editors restore the levels of ABCA4 protein seen in the parental cell line (ABCA4 KI).

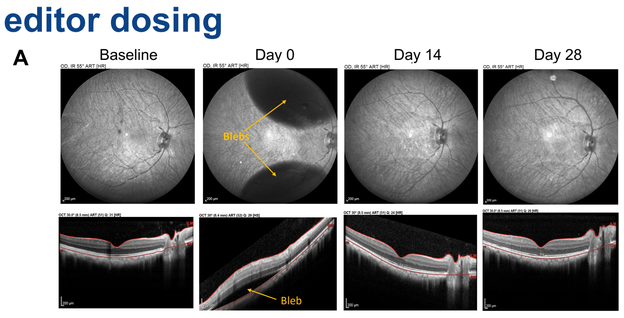
ABCA4 exon editor regulatory elements ensure *in vivo* retinal expression



Exon editor-specific regulatory elements produce robust protein expression in photoreceptors when used to drive GFP expression when dosed subretinally at 1x10^{11} AAV8 vg/100µl injection.

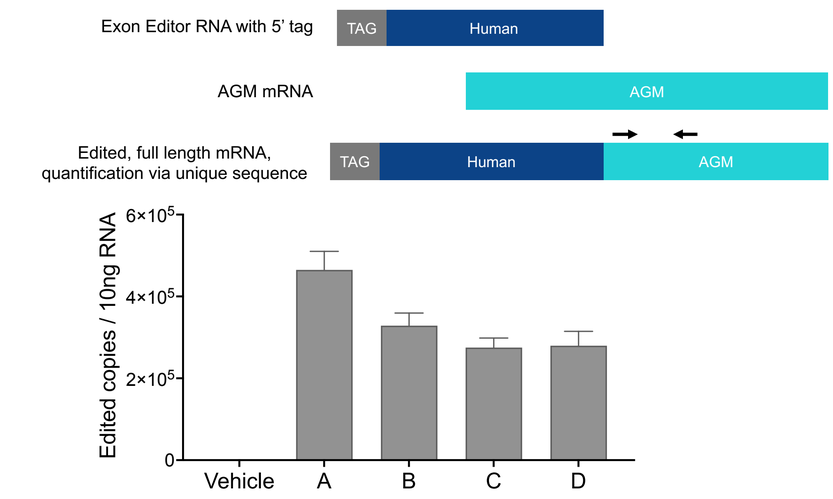
GFP live image (color fundus) and anti-GFP IHC staining of fixed retinal sections.

Preservation of retinal architecture and morphology following subretinal AAV-exon editor dosing



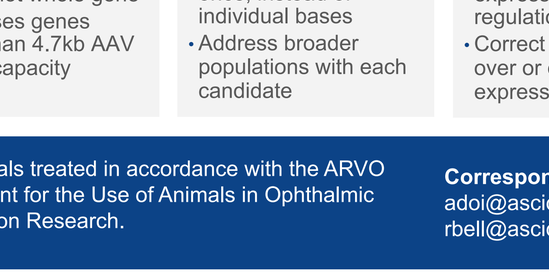
(A) Example of an exon editor treatment: cSLO (confocal scanning laser ophthalmoscope) and OCT (optical coherence tomograph) images of baseline, day 0 post subretinal injections, day 14 and day 28. Post injection the superior and inferior blebs are visible. Each injection was 100µl containing 1x10^{11} vg. A slight 'shadow' of the bleb area remains visible through day 28. (B) Example of day 28 color fundus imaging demonstrating normal retinal morphology in a vehicle and AAV-exon editor treated eye.

Efficient exon editing in the NHP Retina



In the exon editor-treated retina chimeric mRNA was detected with quantitative RT-PCR amplifying the junction between the human coding sequence of the Exon Editor and the African Green Monkey RNA sequence. An example for each exon editor (A, B, C and D) is shown. Sequencing the edited full-length mRNA confirmed the precise edited sequence.

Exon editing produces full length protein



The full-length exon edited mRNA produces a tagged full-length ABCA4 protein. This was detected in the membrane extracts of the retina by western blot of the tag.

An example for each Exon Editor (A, B, C and D) is shown.

Conclusions

While additional work is required to unlock the full therapeutic potential of pre-mRNA Exon Editing, this report demonstrates the viability of pre-mRNA Exon Editing as a therapeutic strategy. The RNA screening platform described here has enabled the discovery of additional novel pre-mRNA Exon Editing molecules for other genetic targets unable to be sufficiently addressed by conventional gene therapy or editing strategies. Results from these screens promise to enable sequence-and structure-based in silico prediction of the performance of synthetic non-coding RNAs. This report highlights the potential of RNA exon editing to treat ABCA4-related retinopathies, and other diseases for which replacement of multiple contiguous exons may provide a novel treatment strategy. Additional studies are ongoing to advance this technology toward the clinic.

Large Genes	High Mutational Variation	Control expression levels
• Replace only damaged exons, not whole gene • Addresses genes larger than 4.7kb AAV capsid capacity	• Edit whole exons at once, instead of individual bases • Address broader populations with each candidate	• Maintain native gene expression levels and regulation • Correct genes where over or off target expression is high risk

All animals treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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