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Dual AAV Vector Strategy for Expression of Large Genes Targeted for Stargardt Disease Gene Therapy Development

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Abstract

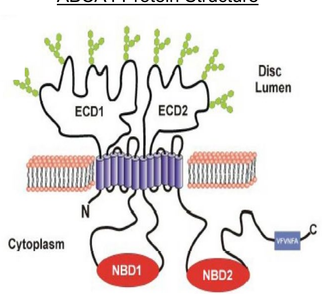
Purpose: Stargardt disease is an inherited, chronic, and progressive retinal dystrophy caused by mutations in the *ABCA4* gene resulting in the most common form of juvenile macular degeneration, for which there are currently no therapeutic options. The ABCA4 coding sequence is 6,822 nucleotides in length and therefore exceeds the packaging capacity of a typical AAV capsid. To enable delivery of large genes, several dual AAV vector strategies have been employed, including homologous recombination, trans-splicing, and intein-mediated protein splicing, but each method has advantages and limitations. To overcome these hurdles, we have created a dual AAV approach that allows efficient recombination of N-terminal and C-terminal ABCA4 fragments by exploiting Cre recombinase.
Methods: The first AAV vector encodes the N-terminal region of the ABCA4 gene and also expresses Cre recombinase via a self-cleaving T2A peptide. The second AAV vector encodes the C-terminal region of the ABCA4 gene. Cre recombinase recognizes LoxP sites inserted into each ABCA4 construct and combines the N- and C-terminal fragments resulting in a full-size ABCA4 expression cassette that will exist episomally within the cell. During the same recombination process, the Cre recombinase gene loses its promoter and inactivates itself.
Results: We have demonstrated that infection in tissue culture with a 1:1 ratio of AAV.ABCA_N and AAV.ABCA_C vectors results in efficient ABCA4 coding sequence reconstitution and full-length ABCA4 protein production within 48 hours. Tissue culture cells infected with ABCA_N and ABCA_C vectors lacking Cre recombinase show no detectable full-length ABCA4 protein after up to 120 hours.
Conclusions: Full-length ABCA4 protein can be efficiently reconstituted from two independent AAV vectors by utilizing Cre recombinase. Future studies exploring Cre-mediated ABCA4 reconstitution *in vivo* and the ability of dual AAV-delivered ABCA4 to clear lipofuscin build-up will be essential milestones to move this early-stage program towards the clinic.

Stargardt disease is the most common form of inherited macular degeneration

- ***Stargardt disease affects 1:8000 to 1:10,000 individuals.***
- Autosomal recessive Stargardt disease is caused by mutations in the *ABCA4* gene.
- Cells responsible for central vision are damaged over time by a buildup of fatty pigments called lipofuscin in the cells within and underlying the macula.
- Symptoms (difficulty seeing in low light, impaired color vision) usually appear in childhood and worsen over time, eventually leading to blindness.

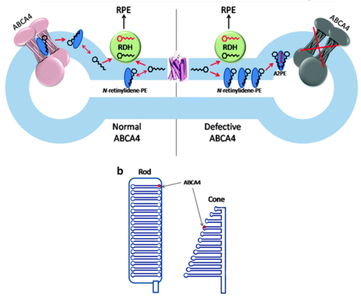
The role of ABCA4 in Stargardt disease

ABCA4 Protein Structure



(Molday, Zhong and Quazi, 2009)

Function of ABCA4 in the visual cycle

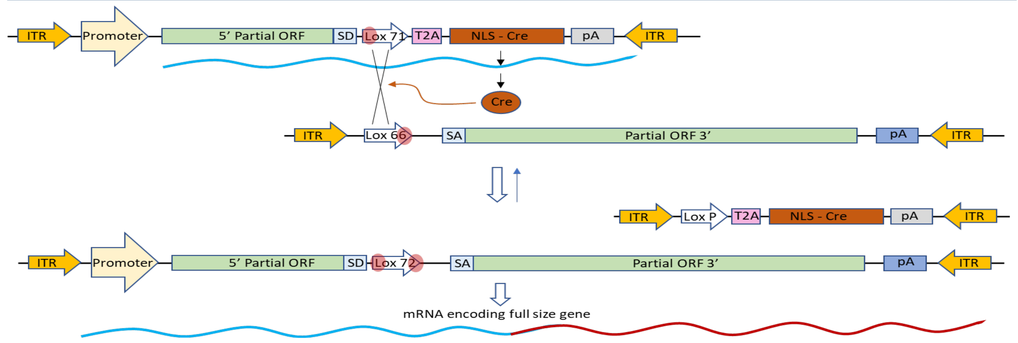


(Tsybovsky, Molday and Palczewski, 2010)

- The *ABCA4* gene encodes a 260kD ABCA4 protein which is a member of the ATP-binding cassette (ABC) transporter family.
- ABCA4 protein is photoreceptor specific and moves N-retinylidene-PE from the intradiscal to the cytoplasmic leaflet of the disc membrane.
- Loss of functional ABCA4 protein leads to the accumulation of retinaldehydes, which become toxic in the photoreceptor and result in photoreceptor death and progressive vision loss.

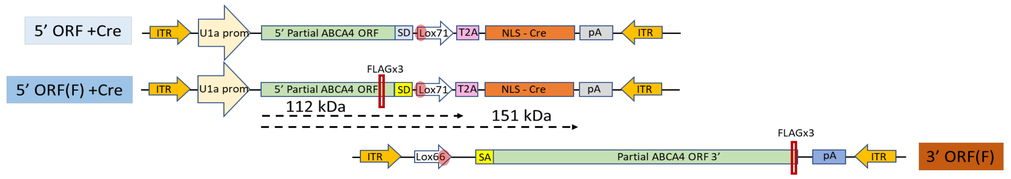
The ABCA4 encoding sequence (6822 nucleotides in length) exceeds the encapsidation capacity of AAV vectors.

Expression of Large Genes Via Dual AAV Utilizing Cre Recombination

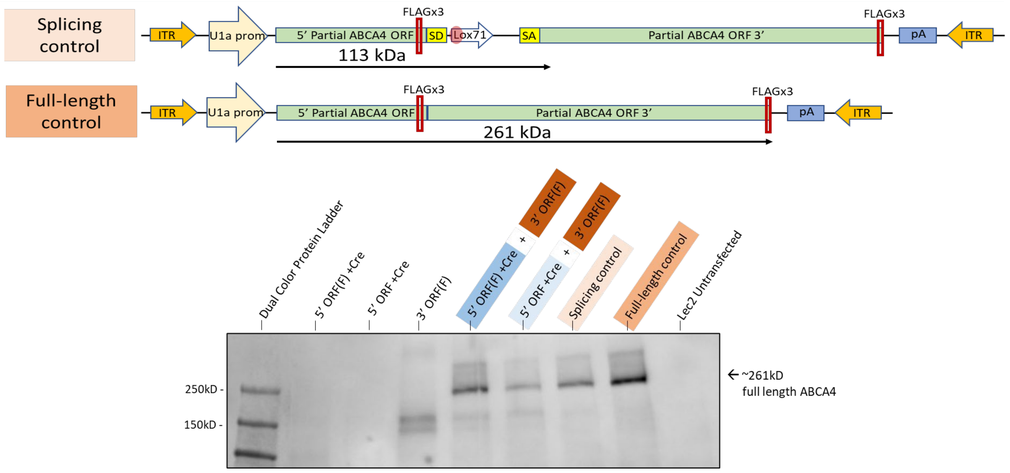


The first AAV vector encodes the 5' partial open reading frame (ORF) of the gene of interest, a splice donor, a modified Lox site, and Cre recombinase ORF preceded by a sequence for a self-cleaving T2A peptide. The second AAV vector encodes the 3' partial ORF of the gene of interest, a splice acceptor and a corresponding modified Lox site. Upon expression of Cre recombinase from the 5' vector, recombination between the two lox sites occurs, resulting in the full-size expression cassette. mRNA splicing will then occur to remove the intron containing lox to produce mRNA encoding the full-size ORF. During the same recombination process, the Cre recombinase gene is inactivated due to loss of its promoter. Modifications on the right or left side of the Lox sites encourages recombination in the forward direction that is ~100x more efficient than recombination in the reverse direction.

ABCA4 Vector Design: Dual ABCA4 Vectors



ABCA4 Vector Design: Full-Length and Splicing Controls

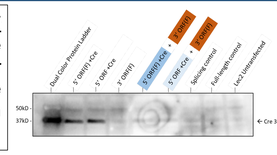


Independent ABCA4 vectors carrying **5' ORF + Cre recombinase** and **3' ORF** successfully undergo recombination and mRNA spicing *in vitro* to produce full-length ABCA4 protein, seen at ~260kD. Differences in band intensity are due to the presence of flag in both vectors (5' + 3') versus a single vector (3' only). **Single ABCA4 vectors** carrying the 5' ORF + Cre or 3' ORF do not produce detectable levels of full-length ABCA4 protein.

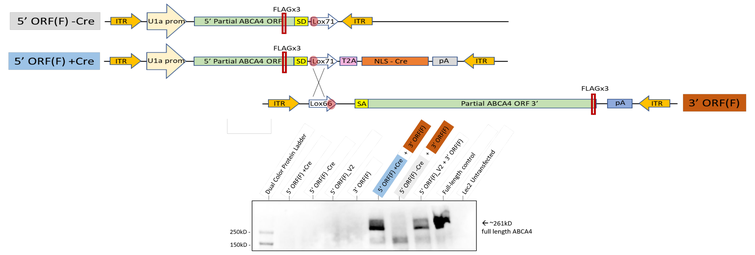
A **full-length control** (oversized construct) containing the complete ABCA4 ORF was used as a positive plasmid control. A **splicing control** containing the full-length ABCA4 ORF interrupted by an intron was used to verify that functional splicing could occur between vector halves. A western blot for FLAG protein detected a ~260kD protein corresponding to full-length ABCA4 present in the full-length and splicing control samples.

Cre Expression is Reduced After Recombination

After recombination of 5' ORF + Cre and 3' ORF ABCA4 vectors, the expression of Cre recombinase protein is significantly reduced compared to the vector containing 5' ORF + Cre alone. This was expected due to Cre losing its promoter during the recombination process.

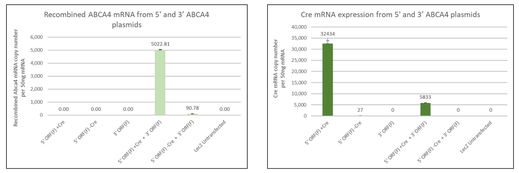


Dual AAV Approach Lacking Cre Shows No Detectable Full-Length ABCA4 Protein



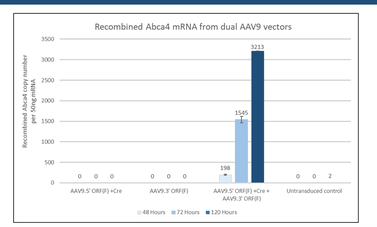
To confirm our results were due to the presence of Cre recombinase in the 5' vector, we created an identical 5' vector lacking Cre. Full-length ABCA4 protein is not detectable from the **dual vectors lacking Cre**.

Quantitation of Full-Length ABCA4 mRNA Before and After Recombination



Dual ABCA4 plasmids utilizing Cre recombinase show a **55x increase** in full-length ABCA4 mRNA synthesis compared to trans-splicing alone. At 48 hours post-transfection, Cre recombinase mRNA levels are **5.5x lower** in dual vector transfected cells compared to single vector transfected cells.

Detection of Full-Length ABCA4 mRNA Using Dual AAV9 Vectors



Recombined Abca4 mRNA from dual AAV9 vectors increases over 120 hours in tissue culture.

Conclusions

- Our dual plasmid system containing 5' and 3' ABCA4 ORFs equipped with Cre recombinase and lox P sites facilitates full-length ABCA4 reconstitution in tissue culture.
- Vector pairs containing Cre recombinase show a **55x increase** in full-length ABCA4 mRNA compared to trans-splicing alone.
- At 48 hours post-transfection, Cre recombinase mRNA levels are **5.5x lower** in dual vector transfected cells compared to single vector transfected cells.
- Dual AAV vectors carrying 5' and 3' ABCA4 fragments equipped with Cre recombinase and lox P sites facilitate full-length ABCA4 reconstitution in tissue culture.