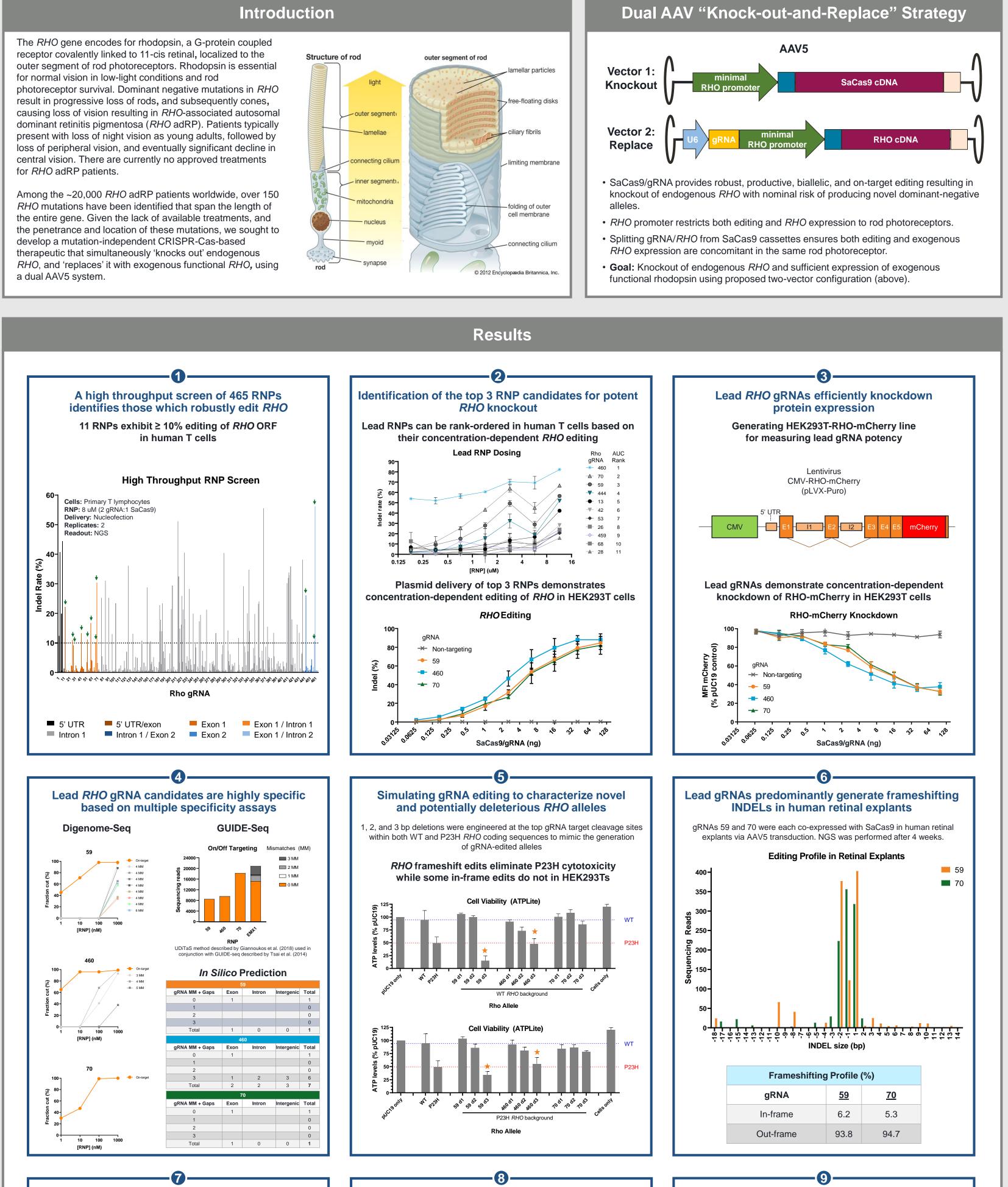# Dual AAV-based 'Knock-out-and-replace' of *RHO* as a Therapeutic Approach to Treat *RHO*-associated Autosomal Dominant Retinitis Pigmentosa (*RHO* adRP)

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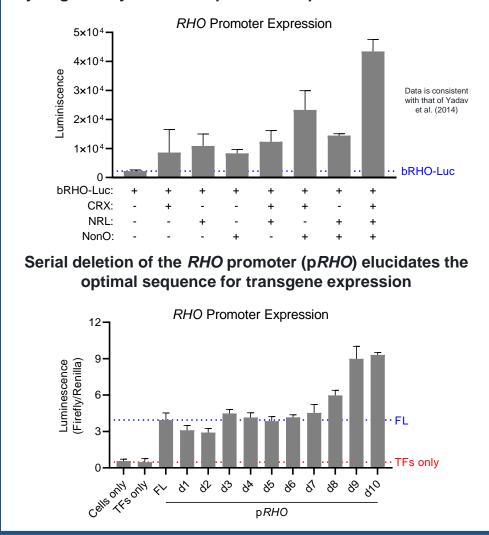
Introduction

The *RHO* gene encodes for rhodopsin, a G-protein coupled receptor covalently linked to 11-cis retinal, localized to the outer segment of rod photoreceptors. Rhodopsin is essential for normal vision in low-light conditions and rod photoreceptor survival. Dominant negative mutations in *RHO* result in progressive loss of rods, and subsequently cones, causing loss of vision in *RHO*-associated autosomal dominant retinitis pigmentosa (*RHO* adRP). Patients typically present with loss of night vision as young adults, followed by loss of peripheral vision, and eventually significant decline in central vision. There are currently no approved treatments for *RHO* adRP patients.

Among the ~20,000 *RHO* adRP patients worldwide, over 150 *RHO* mutations have been identified that span the length of the entire gene. Given the lack of available treatments, and the penetrance and location of these mutations, we sought to develop a mutation-independent CRISPR-Cas-based therapeutic that simultaneously 'knocks out' endogenous *RHO*, and 'replaces' it with exogenous functional *RHO*, using a dual AAV5 system.

Structure of rod / outer segment of rod. © 2012 Encyclopædia Britannica, Inc.

Dual AAV "Knock-out-and-Replace" Strategy

AAV5

Vector 1: Knockout — minimal RHO promoter · SaCas9 cDNA

Vector 2: Replace — U6 · gRNA · minimal RHO promoter · RHO cDNA

- SaCas9/gRNA provides robust, productive, biallelic, and on-target editing resulting in knockout of endogenous *RHO* with nominal risk of producing novel dominant-negative alleles.
- *RHO* promoter restricts both editing and *RHO* expression to rod photoreceptors.
- Splitting gRNA/*RHO* from SaCas9 cassettes ensures both editing and exogenous *RHO* expression are concomitant in the same rod photoreceptor.
- **Goal:** Knockout of endogenous *RHO* and sufficient expression of exogenous functional rhodopsin using proposed two-vector configuration (above).

Results

① A high throughput screen of 465 RNPs identifies those which robustly edit *RHO*

11 RNPs exhibit ≥ 10% editing of *RHO* ORF in human T cells

High Throughput RNP Screen

Cells: Primary T lymphocytes
RNP: 8 uM (2 gRNA:1 SaCas9)
Delivery: Nucleofection
Replicates: 2
Readout: NGS

Legend: 5' UTR · 5' UTR/exon · Exon 1 · Exon 1 / Intron 1 · Intron 1 · Intron 1 / Exon 2 · Exon 2 · Exon 1 / Intron 2

② Identification of the top 3 RNP candidates for potent *RHO* knockout

Lead RNPs can be rank-ordered in human T cells based on their concentration-dependent *RHO* editing

Lead RNP Dosing

Rho gRNA	AUC Rank
460	1
70	2
444	3
13	4
42	5
53	6
26	7
459	8
8	9
28	11

Plasmid delivery of top 3 RNPs demonstrates concentration-dependent editing of *RHO* in HEK293T cells

RHO Editing — gRNA: Non-targeting, 59, 460, 70

③ Lead *RHO* gRNAs efficiently knockdown protein expression

Generating HEK293T-RHO-mCherry line for measuring lead gRNA potency

Lentivirus CMV-RHO-mCherry (pLVX-Puro)

Lead gRNAs demonstrate concentration-dependent knockdown of RHO-mCherry in HEK293T cells

RHO-mCherry Knockdown — gRNA: Non-targeting, 59, 460, 70

④ Lead *RHO* gRNA candidates are highly specific based on multiple specificity assays

Digenome-Seq   **GUIDE-Seq**

On/Off Targeting — Mismatches (MM): 3 MM, 2 MM, 1 MM, 0 MM

UDiTaS method described by Giannoukos et al. (2018) used in conjunction with GUIDE-seq described by Tsai et al. (2014)

In Silico Prediction

59

gRNA MM + Gaps	Exon	Intron	Intergenic	Total
0	1			1
1				0
2				0
3				0
Total	1	0	0	1

460

gRNA MM + Gaps	Exon	Intron	Intergenic	Total
0	1			1
1				0
2				0
3	1	2	3	6
Total	2	2	3	7

70

gRNA MM + Gaps	Exon	Intron	Intergenic	Total
0	1			1
1				0
2				0
3				0
Total	1	0	0	1

⑤ Simulating gRNA editing to characterize novel and potentially deleterious *RHO* alleles

1, 2, and 3 bp deletions were engineered at the top gRNA target cleavage sites within both WT and P23H *RHO* coding sequences to mimic the generation of gRNA-edited alleles

RHO frameshift edits eliminate P23H cytotoxicity while some in-frame edits do not in HEK293Ts

Cell Viability (ATPLite) — WT *RHO* background; WT, P23H

Cell Viability (ATPLite) — P23H *RHO* background; WT, P23H

⑥ Lead gRNAs predominantly generate frameshifting INDELs in human retinal explants

gRNAs 59 and 70 were each co-expressed with SaCas9 in human retinal explants via AAV5 transduction. NGS was performed after 4 weeks.

Editing Profile in Retinal Explants — 59, 70

Frameshifting Profile (%)

gRNA	59	70
In-frame	6.2	5.3
Out-frame	93.8	94.7

⑦ Identification of a minimal *RHO* promoter for driving transgene expression

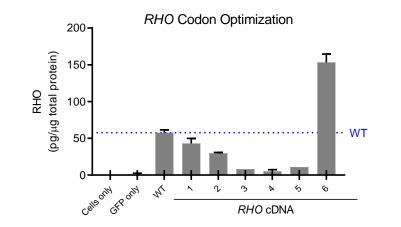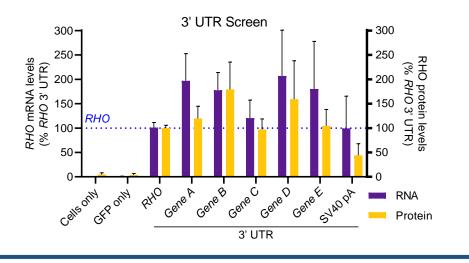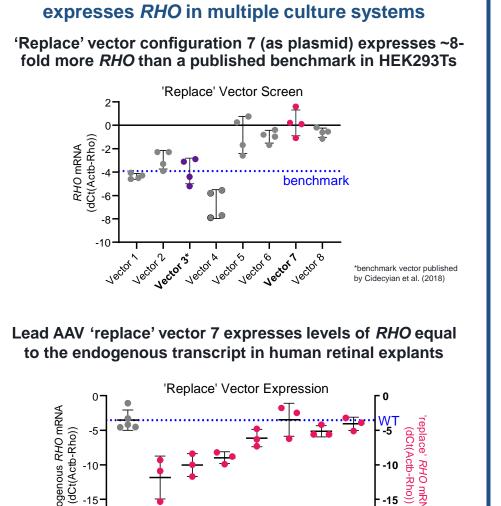**Co-expression of transcription factors CRX, NRL, and NonO synergistically drive *RHO* promoter expression in HEK293Ts**

RHO Promoter Expression

Data is consistent with that of Yadav et al. (2014)

bRHO-Luc · CRX · NRL · NonO

Serial deletion of the *RHO* promoter (p*RHO*) elucidates the optimal sequence for transgene expression

RHO Promoter Expression

⑧ Optimization of cis-acting elements for promoting *RHO* transgene expression

Varying *RHO* cDNA codon usage alters *RHO* transgene expression in HEK293Ts

RHO Codon Optimization

3' UTR elements from heterologous transcripts improve *RHO* transgene expression in HEK293Ts

3' UTR Screen — RNA, Protein

⑨ Top 'replace' vector configuration robustly expresses *RHO* in multiple culture systems

'Replace' vector configuration 7 (as plasmid) expresses ~8-fold more *RHO* than a published benchmark in HEK293Ts

'Replace' Vector Screen — benchmark

*benchmark vector published by Cideciyan et al. (2018)

Lead AAV 'replace' vector 7 expresses levels of *RHO* equal to the endogenous transcript in human retinal explants

'Replace' Vector Expression

Conclusions

- Of 465 RNPs targeting the 5' genetic elements of the *RHO* gene sequence, 11 robustly edit the *RHO* coding sequence.
- 3 lead gRNAs robustly edit endogenous *RHO* loci and knockdown heterologous protein in HEK293Ts in a plasmid concentration-dependent manner.
- 3 lead gRNAs are highly specific to *RHO* by multiple unbiased specificity analyses (Digenome- and GUIDE-seq, *in silico* prediction). Detected off-target sites require verification in follow-up assays.
- Optimized expression vector with minimal *RHO* promoter sequence, optimized coding sequence, and a heterologous 3' UTR element expresses *RHO* transgene higher than the industry benchmark vector.
- AAV5-based delivery of optimized expression vector provides exogenous *RHO* mRNA levels comparable to endogenous *RHO* in human retinal explants.
- These data suggest that it is feasible to develop a dual AAV CRISPR Cas9 based system to knockout and replace rhodopsin in rod photoreceptors.

Acknowledgements