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On RNA Editing—Putting ADAR on Your RADAR

Key Message: Ahead of our Genomic Medicines Days on March 31, 2022, and April 1, 2022, we are publishing a primer on RNA editing. Editing the RNA, i.e., the transcriptome, via ADARs (**A**denosine **D**eaminase **A**cting on **R**NA) is therapeutically desirable when temporary changes are appropriate or when DNA editing is challenging or unpalatable. We envision four drivers in pursuit of RNA editing: (1) established regulatory pathways for constructs that modulate the transcriptome, using well-characterized modalities like ASOs and siRNAs; (2) avoidance of the hitherto unknown consequences of permanent off-target edits to the genome; (3) transient editing, expanding the scope of applications from hepatic and CNS to regenerative medicine; and (4) tunable, targeted dosing based on the desired therapeutic effect. However, the advantages of transcriptome editing should be weighed against some limitations, namely: (1) only half of the SNP mutations at the DNA level accessible by adenine base editors are addressable via RNA editing with the current state-of-the-art technology; and (2) successful uptake of Base editors, a putative one-and-done approach, may erode the attractiveness of RNA editing.

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On the scope of the primer

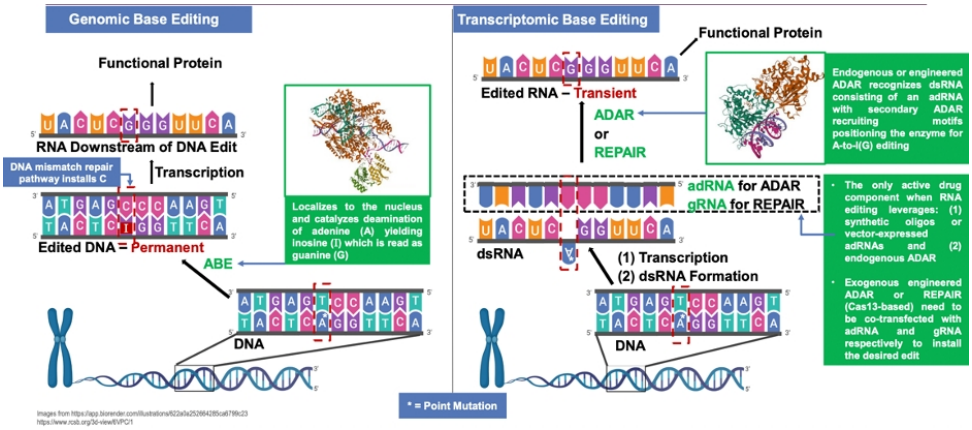
The term RNA editing was introduced into the lexicon in the mid-1980s to describe the insertion or deletion of uridine residues from mitochondrial RNAs of kinetoplastid protozoa. Today, RNA editing can be broadly defined as any site-specific alteration in an RNA sequence that can be copied from the template. These editing processes have been described in organisms from unicellular protozoa to man. The current edition of RNA editing will focus on ADAR's and its potential application to human disease. The A-to-I editing mediated by ADARs can affect several steps during gene expression and regulation, such as splicing, RNA stability, localization, miRNA function. With human applications of RNA editing likely to begin its clinical journey in 2024, the current primer provides a preliminary assessment of the logical targets. Specifically, RNA editing holds intriguing opportunities in key areas: (1) CNS, where ALS, Rett Syndrome, and Genetic Epilepsies each represent multi-billion-dollar markets; (2) Metabolic diseases, including alpha-1-antitrypsin, and GSD1a—in which RNA editing will likely offer complementary strengths and weaknesses compared to CRISPR and Base editors within liver directed targets; (3) Regenerative medicine, the aspirational goal made into a potential reality thanks to the transient nature of RNA editing. In this primer, we cover the essentials for navigating the expanding RNA editing field as well as highlight Private and Public companies at the forefront of this effort.

On the ADAR enzyme—engineered or endogenous—the protein is central to current RNA base editing technology

The RNA editing platforms consist of three primary strategies: (1) **Synthetic oligonucleotides** or a plasmid-expressed RNA vector **adRNA** both employing a mechanism where the construct pairs with the target RNA and recruits an endogenous enzyme called Adenosine Deaminase Acting on RNA (ADAR) to install an A-to-inosine (I); where I is read by translational machinery as G. This approach is akin to adenine base editors (ABEs), albeit leveraging orthogonal enzymology (Figure 1); (2) **Engineered ADAR / adRNA** where the adRNA pairs with the target transcript and is edited with the engineered ADAR; and (3) **Cas13b-based** approach (e.g., REPAIR and RESCUE) where the dead nuclease (dCas13) is fused to the ADAR deaminase domain and, when paired with a guide RNA, localizes to a target transcript and edits a target A-to-I.

See pages 73 - 74 for analyst certification and important disclosures.

Figure 1 - Genomic Base Editing Is Constitutively On While RNA Editing Is Transient

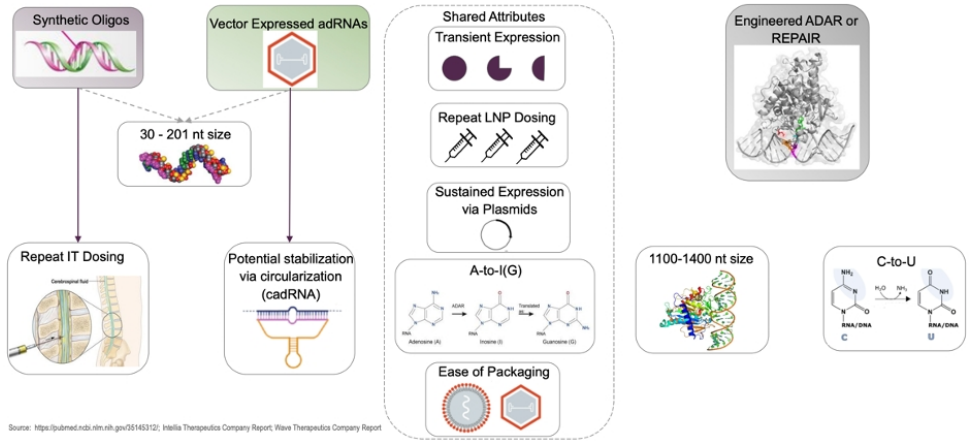


Source: Protein crystal structures were pulled from the protein databank: https://www.rcsb.org/;
DNA art was derived from BioRender: https://biorender.com/;
Guggenheim Securities, LLC.

On the platforms' specifications and applications:

(1) **Size:** Synthetic oligonucleotides (oligos) and adRNAs, 30 to 200 nucleotides; exogenous ADARs, 1,100 to 1,400 nucleotides (amenable for encoding into AAVs); and RESCUE/ REPAIR, ~4,500 nt (challenging for AAV packaging); (2) **Delivery:** The adRNA and exogenous ADARs likely benefit from the same delivery characteristics as ASOs and siRNAs relative to Base and Prime genome editors; (2) **Editing Efficiency:** Synthetic oligo editing efficiency ranges from 30% to 80% depending on the target. Exogenous ADAR editing efficiency ranges from 13% to 80% depending on the construct and target; (3) **Editing Scope:** Synthetic oligos and adRNAs that recruit endogenous ADAR can only access A-to-I(G) edits, whereas exogenous ADAR can be engineered to install both A-to-I(G) and C-to-U edits; and (4) **Therapeutic Applications**: In our opinion, opportunity for RNA base editing spans indications in the CNS/neuro oncology: amyotrophic lateral sclerosis, Rett syndrome, and glioblastoma multiforme. Additionally, indications in metabolic disease and cystic fibrosis look attractive (alpha-1 antitrypsin, glycogen storage disease, and CF mutant subpopulations)

Figure 2 - Oligos, Engineered ADARs, and Cas13b-based Approaches have Complementary and Unique Applications



Source: Katrekar D, et al. Efficient in vitro and in vivo RNA editing via recruitment of endogenous ADARs using circular guide RNAs. Nat Biotechnol. 2022 Feb 10;
NTLA investor presentation;
WVE investor presentation;
Guggenheim Securities, LLC.

On differentiation vs. genome editing

In Biology 101, we are taught the central cellular dogma: (1) DNA replication, (2) transcription of DNA to RNA, and (3) translation of RNA to protein events represent the flow of genetic information for living organisms. *Ipso facto*, drug developers have recognized that the repair of malignant mutations in the DNA represents a potential cure for monogenic diseases by addressing the genetic drivers of downstream disease symptomatology. One-and-done curative genome editing assets are highly attractive from a healthcare perspective, potentially reducing/eliminating symptom management and hospitalizations. However, uncharacterized clinical consequences of genomic off-target editing remain concerning, especially given the novelty of *in vivo* gene editing in humans (EDIT-101 clinical program started 2019 and NTLA-2001 clinical program started 2020). A comparison of genomic and transcriptomic base editing is displayed in Figure 1.

On key stakeholders in RNA editing

In our discussions with key industry participants, the attractiveness of synthetic oligos resonate across multiple domains, including: (1) the regulatory environment that has matured around similar platforms (ASOs and siRNAs); (2) anticipated uptake into a larger, and healthier, population after POC in rare disease; and (3) extensive experience with in-house and CMO-based manufacturing. Interestingly, a handful of companies are exploring adRNA-based RNA editing using endogenous ADAR, including circular adRNAs, for example Shape Tx [Private] and EdiGene [Private]. However, none of the companies we examined have disclosed the implementation of engineered ADAR for the development of RNA-based editing, and we see this platform incubating with academia over the next 1 to 2 years (Table 1). In the public domain, three companies are exploring RNA editing, including BEAM's (2021 10-K) disclosure of the availability of REPAIR and RESCUE for in-house exploration of regenerative medicines. Additionally, WVE has offered a number of examples applying synthetic oligos to address the PiZZ mutational driver in alpha-1 antitrypsin deficiency (A1ATD), compensatory mutations in Rett syndrome, and methodology to disrupt protein-protein interactions. PRQR is also pursuing a synthetic oligonucleotide strategy and has offered a glimpse into RNA editing efficiency for indications such as A1ATD and Usher syndrome. Summary of public companies working in the RNA editing space are summarized in Table 2.

Table 1 - Private Companies Working in the RNA Editing Space

Company	RNA Editing Platform	Funding Round	Amount USD (M)	Date	Partnerships and Investors
ShapeTx	RNAfix / Endogenous ADAR-based editing via vector delivery	Series B	112.00	15-Jul-2021	Partner: Roche (RHHBY) - targets in the Alzheimer, Parkinson, and rare disease areas
		Series A2	35.50	05-Nov-2019	
DTx Pharma	Fatty Acid Ligand Conjugated OligoNucleotide (FALCON™) / Synthetic oligonucleotides	Series B	100.00	01-Mar-2021	Investors: Surveyor, Viva, Access Biotechnology, Comorant Asset Management, Janus Henderson Investors, and Lilly
		Series A2	7.60	06-Jan-2020	
Korro Bio	Oligonucleotide Promoted Editing of RNA (OPERA™) / Synthetic oligonucleotides	Series B	116.00	05-Jan-2022	(1) Founded out of Atlas Venture in 2019 (2) Investors: Eventide Asset Management led the series B with other investors including: Fidelity, Invus, Point72, Verition, Monashee, Sixty Degree Capital, Atlas, NEA, Wu Capital and ~6 others
		Series A	91.50	10-Sep-2020	
ADARx Pharmaceuticals	Not disclosed	Series B	75	08-Sep-2021	Investors: OrbiMed Meathcare Fund Management, Lilly Asia Ventures, SR One, Sirona
LOCANAbio	CORRECTX™ / Broad platform that includes ADAR-based RNA editors, degraders, and exon skipping	Series B	100	12-Dec-2020	Investors: Vida Ventures with participation from: RA Capital Management, Invus, Acuta, and Capital Partners, ARCH Venture Partners, Temasek, Lightstone Ventures, UCB Ventures, and GV
EdiGene	LEAPER™ and LEAPER™ 2.0 / Endogenous ADAR-based editing via vector delivery	Series B2	61.58	21-Apr-2021	Loyal Valley Capital led the Series B other inestors included: BioTrack Capital and Sherpa Healthcare Partners, IDG Capital, Lilly Asia Venture, 3H Health Investment, Huagai Capital, Sequoia Capital China, Alwin Capital and Kunlun Capital
		Series B1	66.78	13-Oct-2020	
		Series A4	11.54	16-Sep-2019	

Source: Guggenheim Securities, LLC.

Table 2 - Public Companies Working in the RNA Editing Space

Company	RNA Editing Platforms	Programs	Development Stage
Wave Life Sciences (WVE)	AIMers - synthetic oligonucleotide with defined stereochemistry and sugar modifications that recruit endogenous ADAR	Alpha-1-antitrypsin deficiency (A1ATD)	Preclinical
		Rett Syndrome – addressing premature stop codons by adding a compensatory R168W mutation to *MECP2*	Preclinical
ProQR (PRQR)	Axiomer® – synthetic oligonucleotides	A1ATD	Preclinical
	PRQR has access to chemistry that confers stereochemical control and sugar modifications	Usher syndrome	Preclinical
Beam Therapeutics (BEAM)	REPAIR – Cas13 fused to deaminase for A-to-I RESCUE – Cas13 fused to deaminase for C-to-U	Not disclosed	Preclinical

Source: Guggenheim Securities, LLC.

On the preliminary comparison of editing efficiency between ADAR-based approaches and base editing

From our analysis of *in vivo* genome editing efficiencies, the base editor VERVE-101 (VERV) has shown the highest turnover, with ~75% editing observed in the liver of NHPs. Comparable, albeit a different target, data from WVE shows ~30% to 50% transcriptome editing in NHPs via a synthetic oligonucleotide system. However, editing efficiency in the transcriptome is not necessarily equivalent to editing in the genome. (1) Genome editing efficiency metrics may be derived from an ensemble cell sample containing both homo and heterozygous cells for the targeted mutation; (2) compensatory transcriptional processes could lead to down-stream changes in the transcriptome that are not reflected in the genomic editing efficiency; (3) the dynamics of translation are not necessarily the same as transcription; (4) biomarker analysis could support genomic vs. transcriptomic editor comparison. See Table 1 for an overview of editing efficiencies for the RNA editors.

Table 3 - Editing Efficiencies Across the RNA Editing Platforms

Construct / Platform	Company or Inventor	On-Target Editing Efficiency
REPAIRv1 / Cas13-based	BEAM	**13%** to **89%** for *Cluc* transcript
REPAIRv2 / Cas13-based	BEAM	**35%** for *Cluc* transcript evaluated in a plasmid library
RESCUEr3 / Cas13-based	BEAM	**15%**
RESCUEr16 / Cas13-based (aka RESCUE)	BEAM	**42%** in HEK293FT cells, 24 different sites including disease-relevant mutation **5%** to **15%** upon multiplex editing
RESCUE-S / Cas13-based	BEAM	**~76%** for the *Gluc* transcript
REPAIR.t1 / Cas13-based	BEAM	**~40%** for *CTNNB1* T41A transcript
REPAIR.t3 / Cas13-based	BEAM	Relatively lower (**~30%**) on-target editing compared to RanCas13b-REPAIR in HeK293FT cells targeting W85X *Cluc* reporter
RESCUE.t1 / Cas13-based	BEAM	**~6%** for the *CTNNB1* transcript (T41I)
RESCUE.t3 / Cas13-based	BEAM	**~6%** for the *CTNNB1* transcript (T41I)
Circ-arRNA151(LEAPER 2.0) / Circular RNA	EdiGene (Private)	**60%-80%** at *GUSB* site 2 in HEK293T cells
Circ-arRNA151_AC50 (LEAPER 2.0) / Circular RNA	EdiGene (Private)	**80%** at *GUSB* site 2 in HEK293T cells
Circ-arRNA151-AΔ14-_AC50 (LEAPER 2.0) / Circular RNA	EdiGene (Private)	**~60%** at PPIA transcript
AIMer / Synthetic oligonucleotide	WVE	**~40%** for *ACTB-33*, primary human retinal pigmented epithelial cells **~60%** for *ACTB-33* primary human hepatocytes **30%** to **50%** (3 constructs) after dosing NHPs subcutaneously (three 5 mg/kg daily doses)
ADAR2-DDC(E488Q, N496F)-λN-P2A-MCP-ADAR2-DDN / Engineered ADAR2	Prashant Mali	**15%** 5'-GAC-3' edited mRNA targeting *RAB7A* transcript
MCP-ADAR2-DD(E488Q, N496F) / Engineered ADAR2	Prashant Mali	**25%** 5'-GAC-3' edited mRNA targeting *RAB7A* transcript
MCP-RESCUEN + RESCUEC-λN / Engineered ADAR2	Prashant Mali	**5%** for 5'-ACG-3' edited

Source: Guggenheim Securities, LLC.

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On RNA Editing
Putting ADAR on Your Radar

March 2022

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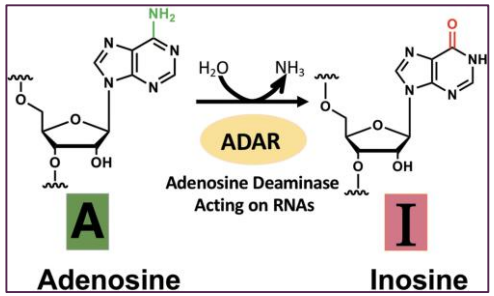
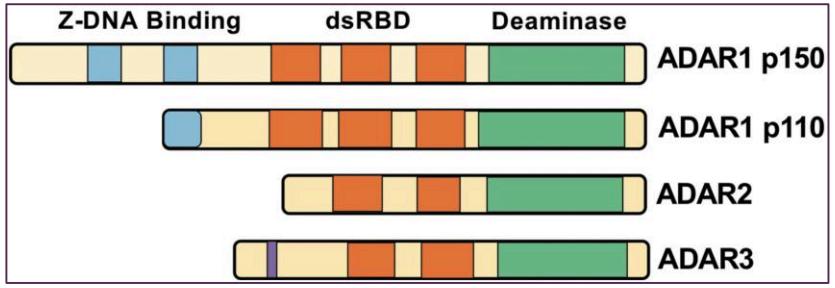
Overview of RNA Editing and Pioneering Companies

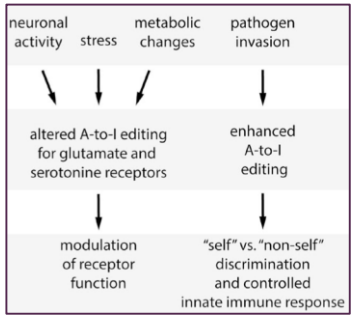
The ADAR Basics



- Adenosine (A) is converted to inosine (I) through hydrolytic deamination of the amino group at the C6 position of adenosine, which is catalyzed by enzymes termed Adenosine Deaminases Acting on RNA (ADARs).
- This reaction requires the formation of a double-stranded RNA (dsRNA) structure in the nucleus. Since inosine contains a similar chemical structure with guanosine (G), inosine base pairs with cytidine instead of uridine.
- Inosines in RNA transcripts are interpreted as guanosines through mRNA-splicing and translation for protein synthesis. As a result, A-to-I modification causes editing of genetic sequences, called A-to-I RNA editing.



- There are three ADAR family members (ADAR1, ADAR2, and ADAR3), and ADAR1 contains two isoforms (p110 and p150).
- All the enzymes contain a conserved deaminase domain, shown in green, and the double-stranded RNA (dsRNA)-binding domain (dsRBD) that determines substrate specificity, shown in orange. ADAR1 p110 and p150 isoforms are different in their Z-DNA-binding domains, shown in blue.
- Only ADAR3 contains an arginine-rich domain, shown in purple.



- A-to-I editing can affect several steps during gene expression and regulation, such as splicing, RNA stability, localization, miRNA function, and translation. A-to-I editing sites mostly reside in noncoding parts of the human transcriptome, such as introns or 3' UTRs, but can also be found in coding regions.
- Interestingly, levels of editing are very diverse and range from barely detectable to almost 100%, depending on the tissue, developmental stage, and substrate. This suggests that editing is dynamically regulated, possibly in response to cellular or extracellular stimuli.

Sources: https://pubmed.ncbi.nlm.nih.gov/30737497/, https://pubmed.ncbi.nlm.nih.gov/33356612/, https://pubmed.ncbi.nlm.nih.gov/35044296/, https://pubmed.ncbi.nlm.nih.gov/29070703/

Endogenous Human ADAR Rewrites the Transcriptome

ADAR1

- **Z-DNA binding domain(s):** (1) Thought to trail a progressing RNA polymerase supporting a targeting mechanism for nascent RNA; (2) potentially implicates ADAR 1 in gene regulation
- **Isoform p110**: Constitutively expressed
- **Isoform p150**: IFN inducible expression and a nuclear export signal for partitioning between the nucleus and cytoplasm
- **Expressed in many tissues**
- **Editing**: A-to-I(G)

ADAR2

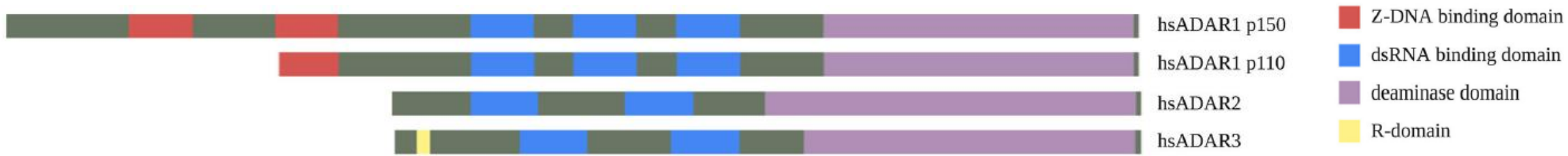
- **Splice variants:** In specific tissues, ADAR2 includes a motif that is similar to ADAR3's R-domain, and it is unknown if this supports single stranded RNA binding
- **Biological functions**: ADAR2 is able to regulate its own activity by generating splice sites within the ADAR2 transcript, yielding variable isoform outputs
- **Expressed in many tissues**
- **Editing**: A-to-I(G)

ADAR3

- **R-domain:** (1) A poly arginine (R) domain that supports single-stranded RNA binding; however, enzyme also can bind to double-stranded RNA; (2) may interact with KPNA2, a nuclear localization signal
- **Editing:** Contains the amino acid residues needed to catalyze adenine deamination in RNA but is editing deficient
- **Expressed exclusively in the brain**
- **Putatively Inactive**

Feature In Common: ADAR1, ADAR2, and maybe ADAR3

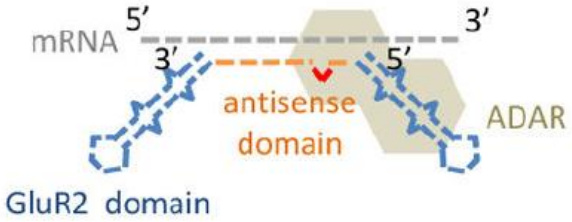
- **ADAR dsRNA size preference**: > 50 bp
- **Deamination of specific As in the target sequence**: Selective deamination of adenosines occurs through loops, bulges and mismatches in the dsRNA
- **DsRNA Localization Motifs:** GluR2
- **DsRNA molecular biology is evolving**: (1) The dsRNA binding domain may facilitate both RNA interaction and homo and hetero protein dimerization (e.g., ADAR1 dsRBD3 can associate with the import factor Transportin-1 (Trn-1), and the complex recognizes a bimodal nuclear localization signal); (2) It is possible that dsRNA binding and homodimerization occurs simultaneously



Sources: https://pubmed.ncbi.nlm.nih.gov/33356612/, https://pubmed.ncbi.nlm.nih.gov/23273215/

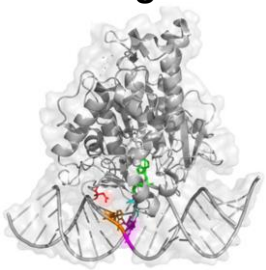
Three Approaches to Leverage ADAR for RNA Editing

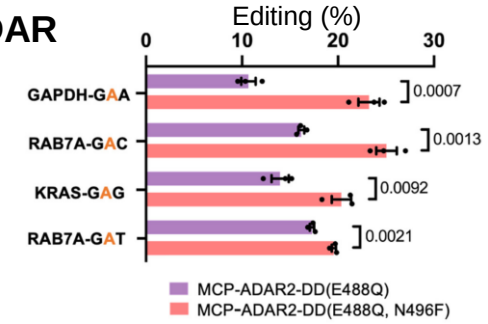
Coopting Endogenous ADAR Pathways



Two primary approaches coopt endogenous Adenosine Deaminase Acting on RNA (ADAR) pathways for installing specific adenosine (A) to inosine (I), read as guanine (G) in RNA: (1) A synthetic RNA oligo such as WVE's AIMer platform or (2) an RNA encoded in and expressed from a plasmid. The construct pairs with target RNA and forms a double stranded RNA (dsRNA). The dsRNA's tertiary appendage (e.g., GluR2), and to a lesser extent sequence, recruits endogenously expressed ADAR containing dsRNA binding domains (dsRBD) and a catalytically active adenosine deaminase domain. Edited mRNA would then enter endogenous translational pathways that yield the desired protein product.

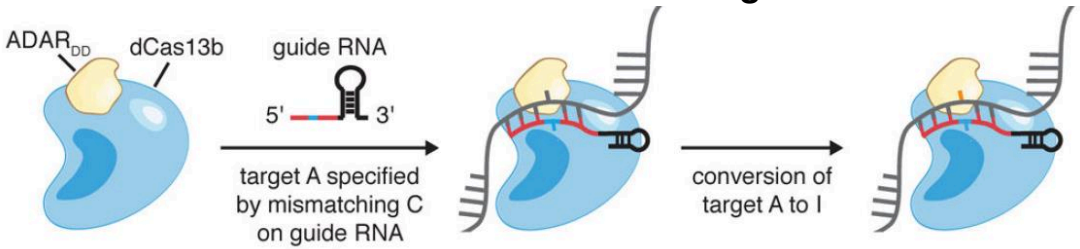
Exogenous Engineered ADAR





Akin to genomic base editing approaches, exogenous ADAR can be used with a guide RNA (adRNA) to target a specific transcript and confer an A-to-I(G) transformation. Recent work has demonstrated that a split engineered ADAR2 approach can be used to dramatically decrease (> 1000-fold decrease relative to earlier generation technology) off-target editing for overexpressed ADAR. The control over engineered ADAR activity needs to be balanced by the additional complexity introduced by delivering a two-component (adRNA and enzyme) therapy.

RNA Guided and Cas13-Based RNA Editing



A two-component system, consisting of (1) engineered ADAR fused to Cas13 and (2) a gRNA, may be used to target single stranded RNA (ssRNA) for editing. Both A-to-I(G) (REPAIR™) and C-to-U(RESCUE™) are accessible, with this platform expanding the RNA base editing space to one that is equivalent to the transition mutations accessible by CRISPR-Cas base editors. The RESCUE platform may install both A-to-I(G) and C-to-U editing, given the platform flexibility to conduct multiplex editing.

Sources: https://pubmed.ncbi.nlm.nih.gov/30737497/, https://pubmed.ncbi.nlm.nih.gov/33356612/, https://pubmed.ncbi.nlm.nih.gov/35044296/, https://pubmed.ncbi.nlm.nih.gov/29070703/

Summary of RNA Editing Oligos and Exogenous RNA Editors

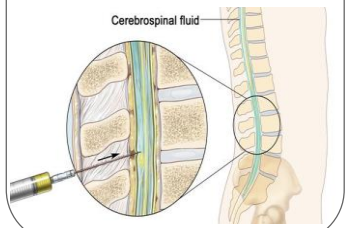
RNA Editing Oligos Recruit Endogenous ADAR

Synthetic Oligos

Vector Expressed adRNAs

30 - 201 nt size

Repeat IT Dosing



Potential stabilization via circularization (cadRNA)

Exogenous RNA Editors (Engineered ADARs or REPAIR)

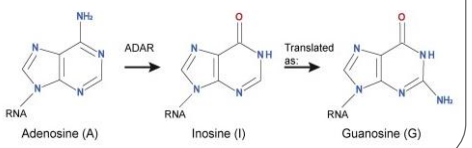
Shared Attributes

Transient Expression

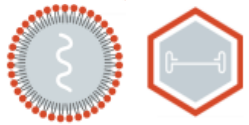
Repeat LNP Dosing

Sustained Expression via Plasmids

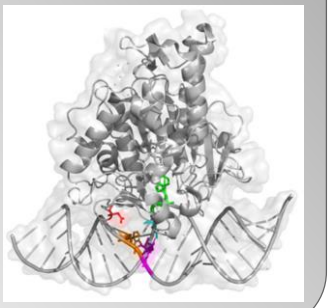
A-to-I(G)



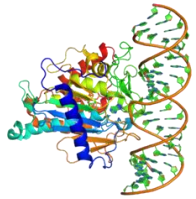
Ease of Packaging



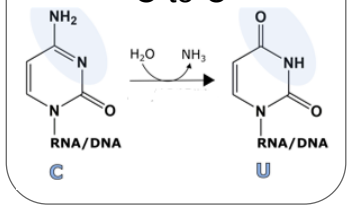
Engineered ADAR or REPAIR



1100-1400 nt size



C-to-U



Sources: https://pubmed.ncbi.nlm.nih.gov/35145312/; Intellia Therapeutics (NTLA) investor presentation; Wave Therapeutics (WVE) investor presentation

Gene Editing = Constitutively On Whereas No RNA Editor = Pharmacological Off Switch

Genomic Base Editing

Functional Protein

RNA Downstream of DNA Edit

Transcription

DNA mismatch repair pathway installs C

Edited DNA = Permanent

ABE

Localizes to the nucleus and catalyzes deamination of adenine (A) yielding inosine (I) which is read as guanine (G)

DNA

Transcriptomic Base Editing

Functional Protein

Edited RNA – Transient

ADAR or REPAIR

Endogenous or engineered ADAR recognizes dsRNA consisting of an adRNA with secondary ADAR recruiting motifs positioning the enzyme for A-to-I(G) editing

adRNA for ADAR

gRNA for REPAIR

dsRNA

(1) Transcription

(2) dsRNA Formation

DNA

- The only active drug component when RNA editing leverages: (1) synthetic oligos or vector-expressed adRNAs and (2) endogenous ADAR
- Exogenous engineered ADAR or REPAIR (Cas13-based) need to be co-transfected with adRNA and gRNA respectively to install the desired edit

* = Point Mutation

Sources: Images from https://app.biorender.com/illustrations/622a0a252664285ca6799c23 and https://www.rcsb.org/3d-view/6VPC/1

The Opportunity and Challenges For RNA Editing

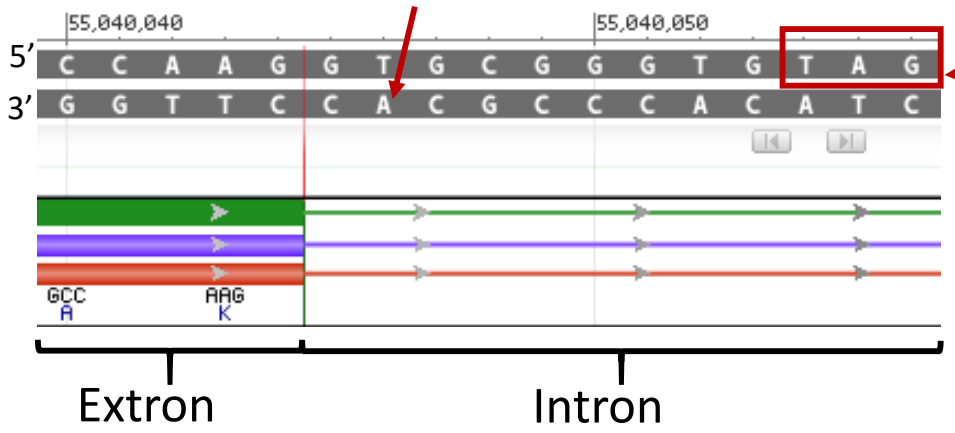
Opportunities:

1. **Base editing shortfalls potentially addressed by RNA editors**: (1) Irreversible guide-dependent and guide-independent off-target editing in the genome; (2) bystander editing; (3) long-term unknown unknowns from editing the genome
2. **Targeted RNA editing can operate in post-mitotic cells:** Expands the repertoire of cells that can be treated. This is particularly important for genetic diseases that are diagnosed after the onset of their symptoms in fully-differentiated cells
3. **Many of the RNA-based approaches rely on human enzymes that will not be recognized as foreign**
4. **Diversity in RNA editing approaches**: There is a spectrum of RNA editing platforms that include: (1) constructs of various sizes, including small synthetic oligos that do not require viral- or lipid nanoparticle-mediated delivery to (2) larger Cas13-based constructs that do not utilize endogenous ADAR pathways to enact RNA editing; additionally (3) engineered ADARS can be leveraged to fine-tune the activity of editing and can fit into AAV vectors
5. **Transient Transcriptome Editing**: Whereas Base editing results in lasting edits to the genome that are carried over the lifetime of an edited cell, RNA editing would support more flexibility in dosing regimen by supporting strategies such as titration and alterations in dosing frequency

Risks

1. **Base editing wins**: Overlapping therapeutic indications being pursued in the genomic Base editing and RNA editing space plays out in favor of Base editing with higher levels of editing and long-term safety data
2. **Off-target editing in the transcriptome**: Unknown Unknowns for off-target editing in the transcriptome and the potential translation to toxic protein products
3. **Dosing frequency in the chronic therapy context**: Durability concerns relative to genomic editing where inconvenient dosing routes and high dosing frequency for RNA editors may erode the attractiveness of developed therapies
4. **Limited in Addressable SNPs**: If the G>A mutation occurs on the template strand, RNA editing (A-to-I editors) cannot restore the transcript to the WT sequence (see PCSK9 targeting example [VERV] on the right)

Targeting PCSK9-1 to the DNA template strand supports protein translational readthrough into the intronic region resulting in termination after three amino acids



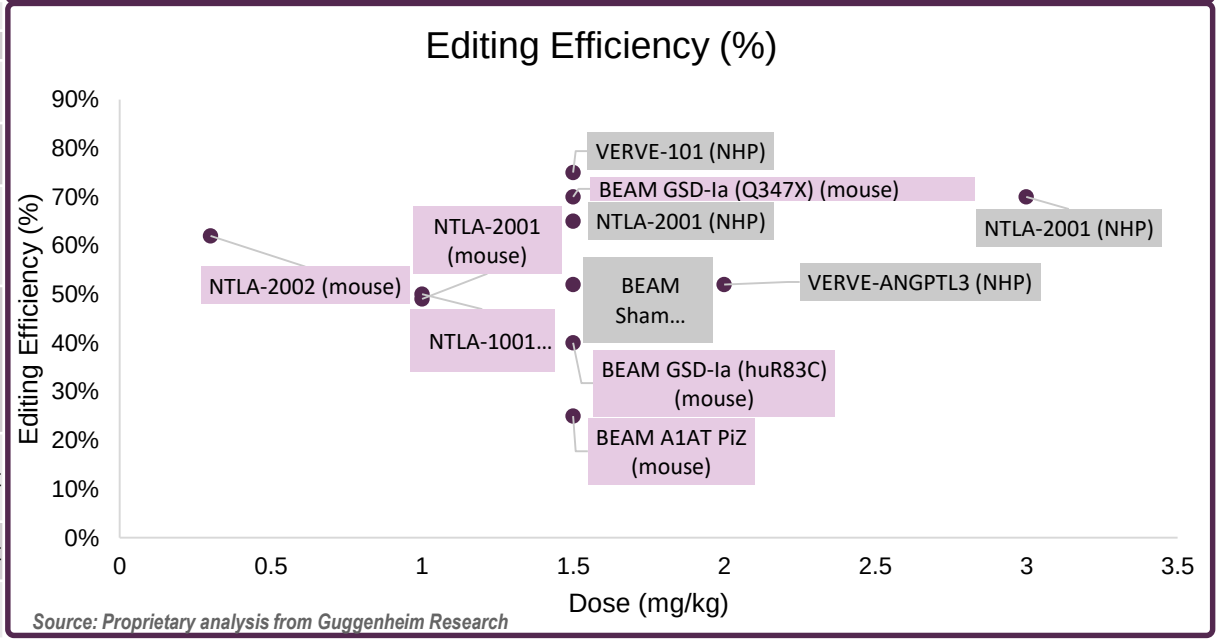
Stop codon on non-template strand results in mutated and truncated PCSK9

Source: https://www.ncbi.nlm.nih.gov/gene/255738

ADAR-Based Approaches Vs. Genomic Based Editing—Proprietary Analysis of Editing Efficiencies

Construct / Platform	Company or Inventor	On-Target Editing Efficiency
REPAIRv1 / Cas13-based	BEAM	**13%** to **89%** for *Cluc* transcript
REPAIRv2 / Cas13-based	BEAM	**35%** for *Cluc* transcript evaluated in a plasmid library
RESCUEr3 / Cas13-based	BEAM	**15%**
RESCUEr16 / Cas13-based (aka RESCUE)	BEAM	**42%** in HEK293FT cells, 24 different sites including disease-relevant mutation **5%** to **15%** upon multiplex editing
RESCUE-S / Cas13-based	BEAM	**~76%** for the *Gluc* transcript
REPAIR.t1 / Cas13-based	BEAM	**~40%** for *CTNNB1* T41A transcript
REPAIR.t3 / Cas13-based	BEAM	Relatively lower (**~30%)** on-target editing compared to RanCas13b-REPAIR in HeK293FT cells targeting W85X *Cluc* reporter
RESCUE.t1 / Cas13-based	BEAM	**~6%** for the *CTNNB1* transcript (T41I)
RESCUE.t3 / Cas13-based	BEAM	**~6%** for the *CTNNB1* transcript (T41I)
Circ-arRNA151(LEAPER 2.0) / Circular RNA	EdiGene (Private)	**60%-80%** at *GUSB* site 2 in HEK293T cells
Circ-arRNA151_AC50 (LEAPER 2.0) / Circular RNA	EdiGene (Private)	**80%** at *GUSB* site 2 in HEK293T cells
Circ-arRNA151-AΔ14-_AC50 (LEAPER 2.0) / Circular RNA	EdiGene (Private)	**~60%** at PPIA transcript
AIMer / Synthetic oligonucleotide	WVE	**~40%** for *ACTB-33,* primary human retinal pigmented epithelial cells **~60%** for *ACTB-33* primary human hepatocytes **30%** to **50%** (3 constructs) after dosing NHPs subcutaneously (three 5 mg/kg daily doses)
ADAR2-DDC(E488Q, N496F)-λN-P2A-MCP-ADAR2-DDN / Engineered ADAR2	Prashant Mali, PhD	**15%** 5'-GAC-3' edited mRNA targeting *RAB7A* transcript
MCP-ADAR2-DD(E488Q, N496F) / Engineered ADAR2	Prashant Mali, PhD	**25%** 5'-GAC-3' edited mRNA targeting *RAB7A* transcript
MCP-RESCUEN + RESCUEC-λN / Engineered ADAR2	Prashant Mali, PhD	**5%** for 5'-ACG-3' edited

Key Message: From our analysis of *in vivo* genome editing efficiencies, the base editor VERVE-101 (VERV) has shown the highest turnover, with ~75% editing observed in the liver of NHPs. Comparable, albeit a different target, data from WVE shows ~30% to 50% transcriptome editing in NHPs *via* a synthetic oligonucleotide system. **Comp Factors for Consideration:** Editing efficiency in the transcriptome is not necessarily equivalent to editing in the genome. (1) Genome editing efficiency metrics may be derived from an ensemble cell sample containing both homo and heterozygous cells for the targeted mutation; (2) compensatory transcriptional processes could lead to down-stream changes in the transcriptome that are not reflected in the genomic editing efficiency; (3) the dynamics of translation are not necessarily the same as transcription; (4) biomarker analysis could support genomic vs. transcriptomic editor comparison.



Source: Proprietary analysis from Guggenheim Research

SWOT Endogenous ADAR Editing

	Positives	Unknowns
Internal Origin	**Strengths:** • **Single active component dosed**: (1) Technology around the delivery of synthetic oligos is rapidly expanding with mature clinical and regulatory exposure; (2) endogenous expression of adRNA simplifies delivery and represents a construct that easily fits into LNPs or AAVs • **Cyclization leads to longer stability**: RNA cyclization approach's can be used to extend the lifetime of vector-expressed adRNA relative to linear constructs	**Weaknesses:** • **Dose titration control**: The amount of endogenous ADAR cannot be controlled and relies upon endogenous ADAR expression levels, which may be subject to temporal changes • **Unintended reading of inosine**: There is *in vitro* data using rabbit-based systems that suggest inosine is not strictly read as guanine
External Origin	**Opportunities:** • **Genome editing safety profile concerns may not be addressed within the next 5 to 10 years**: Long-term clinical safety data for gene editing technology may be more than 10 years away, in our opinion. Furthermore, it is widely accepted that gene editing and off-target editing are permanent alterations for persistent cell populations • **Base editors can be too large to fit into AAVs**	**Threats:** • **Genome editing approaches eventually are demonstrated to be safe and effective**: Safe and effective gene editing approaches imply a minimal dosing paradigm with one-and-done well within the realm of reality. Relative to a chronic dosing paradigm needed for exogenous ADAR gene editing may become a preferred approach • **Non-editing approaches**: Small or macromolecular approaches that work down-stream of the pathways being targeted by RNA editing therapies

SWOT Exogenous Engineered ADAR Delivery

	Positives	Unknowns
Internal Origin	**Strengths:** • **Protein and adRNA can be optimized**: The potency and specificity of ADAR can be tuned through mutant screening and guide strand optimization • **Dose titration control**: The amount of engineered ADAR editor delivered to the biological system can be controlled as opposed to a coopting approach that relies upon endogenous ADAR expression levels and temporal changes	**Weaknesses:** • **Redosing:** Unless a constitutive expression platform is used (e.g., Zolgenzma in SMA), redosing would be needed • **Immunological reactions**: We have not encountered data that evaluate the feasibility of chronic engineered ADAR dosing from an immunological perspective
External Origin	**Opportunities:** • **Genome editing safety profile concerns may not be addressed within the next 5 to 10 years**: Long-term clinical safety data for gene editing technology may be more than 10 years away, in our opinion. Furthermore, it is widely accepted that gene editing and off-target editing are permanent alterations for persistent cell populations • **Base editors can be too large to fit into AAVs**	**Threats:** • **Genome editing approaches eventually are demonstrated to be safe and effective**: Safe and effective gene editing approaches imply a minimal dosing paradigm with one-and-done well within the realm of reality. Relative to a chronic dosing paradigm needed for exogenous ADAR gene editing may become a preferred approach • **Non-editing approaches**: Small or macromolecular approaches that work down-stream of the pathways being targeted by RNA editing therapies

SWOT Cas13-Based RNA Editing Approaches

	Positives	Unknowns
Internal Origin	**Strengths:** • **Orthogonal to endogenous ADAR pathways**: It is not likely that RESCUE and REPAIR interfere with endogenous ADAR pathways • **Accesses both A-to-I(G) and C-to-U**: RESCUE can recapitulate all of the transition mutations accessible with base editing	**Weaknesses:** • **Large size**: At ~ 4.5 kb the RESCUE platform may encounter AAV-based delivery challenges • **Redosing**: As editing the transcriptome is transient, Cas13-based RNA editing approaches such as RESCUE and REPAIR will require (1) platforms that can be dosed multiple times; (2) approaches that lead to sustained editing; and/or (3) focus on diseases requiring acute transcriptome editing
External Origin	**Opportunities:** • **Growing uptake of Cas-based systems**: Beam Therapeutics has in-licensed both the RESCUE and REPAIR technologies for potential use in regenerative medicine as indicated in its 2021 Form 10-K • **Full engineering control over the construct:** Excluding endogenous ADAR pathways, RESCUE and REPAIR platforms can be fully engineered whereas other approaches are subject to endogenous expression levels of ADAR	**Threats:** • **Genome editing approaches eventually are demonstrated to be safe and effective**: Safe and effective gene editing approaches imply a minimal dosing paradigm with one-and-done well within the realm of reality. Relative to a chronic dosing paradigm needed for exogenous ADAR gene editing may become a preferred approach • **Non-editing approaches**: Small or macromolecular approaches that work down-stream of the pathways being targeted by RNA editing therapies

Emerging Privates With a Focus on RNA Editing

Company	RNA Editing Platform	Funding Round	Amount USD (M)	Date	Partnerships and Investors
ShapeTx	RNAfix / Endogenous ADAR-based editing via vector delivery	Series B	112.00	15-Jul-2021	Partner: Roche (RHHBY) - targets in the Alzheimer, Parkinson, and rare disease areas
		Series A2	35.50	05-Nov-2019	
DTx Pharma	Fatty Acid Ligand Conjugated OligoNucleotide (FALCON™) / Synthetic oligonucleotides	Series B	100.00	01-Mar-2021	Investors (private unless specified otherwise): Friedman Bioventure Fund, Surveyor capital, Viva BioInnovator (Viva Biotech Holdings, 1873.HK), Access Biotechnology, Cormorant Asset Management, Janus Henderson Investors, Logos Capital, ExSight Ventures, and Eli Lilly (LLY)
		Series A2	7.60	06-Jan-2020	
Korro Bio	Oligonucleotide Promoted Editing of RNA (OPERA™) / Synthetic oligonucleotides	Series B	116.00	05-Jan-2022	(1) Founded out of Atlas Venture (private) in 2019 (2) Investors (private unless specified otherwise): Eventide Asset Management led the series B with other investors including: Fidelity Management & Research Company, Invus, Point72, Verition Fund Management, Monashee Investment Management, Sixty Degree Capital, Atlas Venture, NEA, Wu Capital and ~6 others
		Series A	91.50	10-Sep-2020	
ADARx Pharmaceuticals	Not disclosed	Series B	75	08-Sep-2021	Investors (private unless specified otherwise): OrbiMed Healthcare Fund Management, Lilly Asia Ventures, SR One Capital Management, Sirona Capital
LOCANAbio	CORRECTX™ / Broad platform that includes ADAR-based RNA editors, degraders, and exon skipping	Series B	100	12-Dec-2020	Investors (private unless specified otherwise): Vida Ventures with participation from: RA Capital Management, Invus, Acuta Capital Partners, ARCH Venture Partners, Temasek, Lightstone Ventures, UCB Ventures, and GV
EdiGene	LEAPER™ and LEAPER™ 2.0 / Endogenous ADAR-based editing via vector delivery	Series B2	61.58	21-Apr-2021	Loyal Valley Capital (private) led the Series B other investors included (private unless specified otherwise): BioTrack Capital and Sherpa Healthcare Partners, IDG Capital, Lilly Asia Venture, 3H Health Investment, Huagai Capital, Sequoia Capital China, Alwin Capital and Kunlun Capital
		Series B1	66.78	13-Oct-2020	
		Series A4	11.54	16-Sep-2019	

Sources: FactSet; ADARx, https://www.businesswire.com/news/home/20210908005168/en/ADARx-Raises-75-Million-to-Advance-Growing-Pipeline; LOCANAbio, https://locanabio.com/press-releases/locanabio-announces-100-million-series-b-financing-to-advance-portfolio-of-novel-rnatargeted-gene-therapies-for-neurodegenerative-neuromuscular-and-retinal-diseases/

Public Companies Exploring RNA Editing

Company	RNA Editing Platforms	Programs	Development Stage
Wave Life Sciences (WVE)	AIMers - synthetic oligonucleotide with defined stereochemistry and sugar modifications that recruit endogenous ADAR	Alpha-1-antitrypsin deficiency (A1ATD)	Preclinical
		Rett Syndrome – addressing premature stop codons by adding a compensatory R168W mutation to *MECP2*	Preclinical
ProQR (PRQR)	Axiomer® – synthetic oligonucleotides PRQR has access to chemistry that confers stereochemical control and sugar modifications	A1ATD	Preclinical
		Usher syndrome	Preclinical
Beam Therapeutics (BEAM)	REPAIR – Cas13 fused to deaminase for A-to-I RESCUE – Cas13 fused to deaminase for C-to-U	Not disclosed	Preclinical

Sources: https://wavelifesciences.com/, https://www.proqr.com/, https://beamtx.com/

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Introduction Into RNA Editing Therapeutic Applications

Defining the Scope of RNA Editing—CNS Holds Intriguing Opportunities

Indication	Opportunities or Limitations for an RNA Editing Approach	RNA Target	U.S. Market Estimated Number of Patients	Standard of Care
Amyotrophic lateral sclerosis	Sporadic ALS patients show downregulation of ADAR2 in spinal motor neurons with concomitant lowering of GluA2 Q/R editing (https://pubmed.ncbi.nlm.nih.gov/22226999/)	Restore RNA A-to-I for GluA2 subunit of the mammalian AMPA receptor at the Q/R site	12,000 to 29,000 ~5,000 new diagnoses per year	- Rilutek® (riluzole) [ticker SNY] – preferentially blocks tetrodotoxin-sensitive sodium channels - Radicava® (edaravone) [ticker MTZPY] – unknown MOA: extends survival and/or time to tracheostomy - Disease is ultimately fatal
Rett syndrome	Genetically-defined disease with high unmet medical need	c.502C>T, p.(Arg168*) nonsense mutation	~1,000 to 2,000 girls The c.502C>T mutation is ~8% of the Rett population (1 in 10,000 girls)	Multisystem comorbidities evolve throughout the lifespan. No approved therapies for Rett The syndrome can go undiagnosed or misdiagnosed which makes patient identification difficult (https://rarediseases.org/rare-diseases/rett-syndrome)
Epilepsy Developmental epileptic encephalopathy (DEE)	Clinical biopsy data offer an ambiguous picture of RNA editing's utility in the epileptic brain (https://pubmed.ncbi.nlm.nih.gov/33393416/) Current A-to-I RNA editing technology is not applicable for the Q607E *GRIA2* mutation as repair would require the transverse C-to-G mutation in DEE	The molecular mechanism of epileptogenesis arising from the defect of GluA2 Q/R site editing may hold future therapeutic opportunities. However, current data do not support a clear target prosecution strategy GluA2 Q/R editing in clinical specimens of epilepsy patients are inconsistent	3.4 million	Many small molecules that inhibit pathways leading to excitatory neuronal activity or enhance inhibitory neuronal signaling (https://www.nature.com/articles/s41467-019-10910-w)
Glioblastoma multiforme	GluA2 editing deficits leads to Akt phosphorylation, thereby promoting cellular proliferation and mobility	Restore RNA A-to-I for GluA2 subunit of the mammalian AMPA receptor at the Q/R site	5,000 to 6,000	Alkylating agents, mainly temozolomide Median survival time for patients with newly diagnosed GBM is 9.7 months
Anxiety, depression, bipolar disorder, and schizophrenia	Five ADAR editing sites have been identified for the 5-HT_{2C}R transcript leading to 24 protein variants	Serotonin receptor (5-HT_{2C}R)	Expansive population (e.g., ~3.7 M in schizophrenia)	Multiple therapies available dependent upon the condition

Sources: https://pubmed.ncbi.nlm.nih.gov/22226999, https://pubmed.ncbi.nlm.nih.gov/33393416/, https://rarediseases.org/rare-diseases/rett-syndrome, https://www.nature.com/articles/s41467-019-10910-w

Defining the Scope of RNA Editing—Metabolic Diseases and CF Subpopulations

Indication	Opportunities or Limitations for an RNA Editing Approach	RNA Target	U.S. Market Estimated Number of Patients	Standard of Care
Alpha-1-antitrypsine deficiency	RNA editing represents a potential hedge – BEAM approach shows bystander D341G editing of unknown consequence	Genomic base editing to correct E342K of SERIPINA1 (BEAM) HDR genomic insertion or PiZZ knockout (NTLA): NTLA-2003 and NTLA-3001 PiZZ mutation (E342K) of SERIPINA1 / RNA Editing (WVE)	PiZZ Lung in Caucasians: 50,000 - 100,000 PiZZ Liver in Caucasians: 50,000 - 100,000	Augmentation therapy for slowing the progression of lung manifestations: ~$78,500 USD / year (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5392996/pdf/12931_2017_Article_543.pdf) No therapy for liver manifestation
Glycogen storage disease 1a	Accessible mutation for RNA editing (G>A occurs on non-template strand of DNA) Potential hedge for BEAM-301	Glucose-6-phosphatase gene (R83C) (e.g., BEAM-301 for genomic base editing)	~300 (1/100,000 live births with 80% carrying the R83C mutation) (https://ojrd.biomedcentral.com/articles/10.1186/1750-1172-6-27)	Dietary supplementation of cornstarch several times per day and during the night
Heterozygous familial hypercholesteremia	Not applicable using the PCSK9-1 (VERV) approach. PCSK9-1 targets splice donor at the boundary of *PCSK9* exon 1 and intron 1 by mutating the template DNA strand (A-to-I)	Transcript targeting strategy analogous to PCSK9-1 is not accessible via RNA editing	NA	NA
Cystic fibrosis	There are CF mutations outside of the major variant F508del (F508del representing ~70% of the CF population and not addressable via RNA editing) that may be addressable through genomic or transcriptomic base editing	G551D (c.1652G>A) accessible G>A on non-template strand W1282X (c.3846G>A) accessible G>A on non-template strand	G551D (2.10% of CF) W1282X (1.22% of CF) 2,000-3,000 (https://pubmed.ncbi.nlm.nih.gov/34175042/)	G551D: Kalydeco, Symdeko, Trikafta (VRTX) W1282X: No approved treatment
Regenerative medicine	Potentially broad indication, targets not well-defined	BEAM mentioned this approach in its 2021 10-K (BEAM)	NA	NA

Sources: https://ojrd.biomedcentral.com/articles/10.1186/1750-1172-6-27, https://pubmed.ncbi.nlm.nih.gov/34175042/, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5392996/pdf/12931_2017_Article_543.pdf

Our Opinion on Therapeutic Applications for RNA Editing

Disease and Approach	Advantages / Disadvantages	Hurdles
Achievable • **Glioblastoma multiforme**: Intrathecally delivered ADAR-recruiting synthetic oligo targeting GluA2 Q/R restoration in transcriptome • **Amyotrophic lateral sclerosis:** Intrathecally delivered ADAR-recruiting synthetic oligo targeting GluA2 Q/R restoration in transcriptome	**Unmet need**: Both indications represent diseases with high unmet medical need **Development path**: Regulatory pathways and dosing rationale for intrathecally delivered synthetic oligonucleotides (e.g., Spinraza (nusinersen) by BIIB) have been established **Limited scientific knowledge**: Only preliminary clinical evidence support the utility of prosecuting these pathways *in vivo*	**Dosing**: Chronic dosing regimens may limit the utility of the therapies depending on frequency of administration **Distribution in the CNS**: Preclinical models will not necessarily be predictive for the therapeutically-relevant distribution of synthetic oligonucleotides in the human CNS compartment
Feasible • **Alpha-1-antitrypsine deficiency (A1ATD)**: Repair the *SERPINA1 PiZZ* transcript effectively restoring the expression of WT A1ATD • **Glycogen Storage Disease 1a (GSD1a)**: Targeting the mutational driver of the R83C variant	**Target rationale (both)**: Well-defined monogenic drivers **Preclinical POC**: Both targets have demonstrated POC with RNA and/or genomic base editing examples available **Incomplete target editing in A1ATD**: Although target A1ATD restoration levels have been proposed for addressing lung manifestations of the disease (~ 11 µM in circulation) it is unclear the target PiZ lowering in the liver needed to restore / preserve hepatocyte health	**Dosing**: Chronic dosing may limit the utility of the therapies depending on frequency of administration. However, the development of subcutaneous at-home delivery may prove to be a viable path forward **Variability**: The target of A1ATD is the liver. Effective delivery of A1ATD RNA editing therapies to the liver may face challenges due to heterogeneity in liver cell heath and LDL receptor expression
Aspirational • **Epilepsy / Developmental epileptic encephalopathy** • **Schizophrenia** • **Bipolar**	**Path forward is ambiguous**: (1) Epilepsy, GluA2 Q/R editing in clinical specimens of epilepsy patients are inconsistent (2) Schizophrenia and bipolar disorder, serotonin receptor (5-HT_{2C}R) biology is influenced by ADAR-based editing. However, how specific edits in the transcriptome may modulate the disease are not well understood	**Dosing**: Chronic dosing may limit the utility of the therapies depending on frequency of administration **Availability of therapies**: In epilepsy there are a number of approved pharmacological and surgical approaches that may be used to treat the disease, creating a crowded competitive landscape

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RNA Editing—The Basics

RNA Editing—The Basics

RNA editing refers to any RNA site-specific substitution. RNA editing can modify the primary structure of the proteins by introducing:

- Single amino acid substitutions
- New start and stop codons
- Modifying splicing sites
- It can also affect RNA stability by modifying the Untranslated Regions (UTRs)

Adenosine to inosine (A-to-I), RNA base editing, is the most frequent sequence modification, widespread in the majority of human genes:

- The conversion of A-to-I is mediated by enzymes named Adenosine Deaminases Acting on RNA (ADAR)
- ADAR enzymes are able to bind to double stranded RNA (dsRNA) and modify A nucleotide into I by deamination

The mammalian genome encodes for three members of the ADAR family:

- ADAR1 (encoded by the gene ADAR)
- ADAR2 (encoded by ADARB1) and ADAR3 (encoded by ADARB2)
- ADAR1 and -2 are active enzymes, whereas ADAR3 lacks the enzymatic activity and is mainly expressed at low-level in brain regions

ADAR enzymes edit millions of sites in the mammalian transcriptome. The majority of these sites are located in non-coding sequences: 5′UTRs, 3′UTRs and in intronic retrotransposons, such as ALU-inverted repeats:

- In principle, RNA editing in repetitive elements, such as ALU sequences, is primarily mediated by ADAR1
- RNA editing at the coding sites (namely re-coding sites) is preferentially performed by ADAR2
- However, a certain degree of overlap exists between target sites of the two enzymes
- About 2.5 million editing sites have been identified up to now and are deposited in public databases

The ADAR Basics-2-Functionality

- Loss of ADAR1 leads to embryonic lethality in mice that is accompanied by liver disintegration and a dramatic increase in interferon signaling
- This phenotype can be rescued by the deletion of cytoplasmic RNA sensors Mda5 or MAVS, strongly suggesting that in the absence of ADAR1-mediated RNA editing, endogenous RNAs are interpreted as foreign or viral by the innate antiviral immune system
- Interestingly, ADAR1 can also prevent inadvertent activation of PKR-mediated shut-down of translation, suggesting that ADAR1 is a general modulator of RNA-sensing pattern recognition receptors. Historically, IFN cancer therapies have been hampered by tumor resistance to its anti-proliferative effects. Inhibiting ADAR1 during IFN therapy could lead to PKR activation and subsequent cell growth arrest, thereby enhancing the anti-proliferative effects and efficacy of IFN therapy

- ADAR2 is most strongly expressed in the nervous system but also in some organs such as the intestine and the vasculature
- Loss of ADAR2 in mice leads to lethality around three weeks post-partum, which is accompanied by epileptic seizures
- Interestingly, a knock-in of a pre-edited version of the glutamate receptor subunit 2 Gria2 can rescue this lethality, indicating that Gria2 is a major substrate of ADAR2
- Still, ADAR2 also edits many other RNAs in their non-coding regions but also in their coding regions, which frequently leads to protein recoding. These protein recoding events alter protein function and therefore affect cellular and organismic physiology

- The catalytically inactive ADAR3 is expressed primarily in the brain at a relatively low level and its loss has been associated with minor learning alterations in mice

Sources: https://pubmed.ncbi.nlm.nih.gov/30737497/, https://pubmed.ncbi.nlm.nih.gov/33356612/, https://pubmed.ncbi.nlm.nih.gov/35044296/, https://pubmed.ncbi.nlm.nih.gov/29070703/

The ADAR Basics-3—Editing

All ADARs contain two main structural motifs:

- The first is the double stranded RNA Binding Domain (dsRBD). dsRBDs are ADARs' main targeting domain, recognizing complicated higher order structures within RNAs. They can bind to perfect duplex RNA, though they will also bind to imperfect structures with bulges, hairpins and mismatches with even higher affinity
- The natural RNA structures that ADARs recognize are diverse. Because no defining motifs have been identified, it has not been possible to predict which RNAs will be recognized by ADAR based on their sequence or structural characteristics
- The main function of dsRBDs is to guide ADAR's catalytic activity to its target adenosine

The second structural motif, termed the Deaminase Domain (DD), carries out the catalytic activity:

- The crystal structure of the DD has been solved which, coupled with functional studies on ADAR and other nucleotide deaminases, have made it clear that ADAR uses a base flipping mechanism to move the adenosine out of the A-form RNA helix, and into the enzyme's catalytic pocket, in order to catalyze A-to-I(G) conversion

The efficiency at which the DD edits a specific target A is highly affected by neighboring bases:

- For example, nearest neighbor preferences dictate that editing will be efficient when the 5' neighbor is a uracil (U) and poor when it is a G. On the 3' side, however, a G is preferred over a U

Genomic Variation and Regulation of ADAR

- ADAR-mediated RNA editing is tightly controlled and regulated to assure cellular and organismic homeostasis
- Protein-recoding RNA-editing events vary largely between tissues and throughout development. For instance, in mammals, editing patterns are very low during embryonic development but increase rapidly post-partum. Moreover, some targets show high editing patterns in a tissue-specific manner
- The abundance and conservation of genomic repeats is a major regulator of RNA editing. As inverted repeats within transcripts are major sites of recruitment for ADARs, the repeat repertoire in transcripts not only controls the editing levels of the repeats but also of the adjacent sites
- ADAR1 plays an important role in the attenuation of IFN induction by editing and thereby “masking” self-dsRNAs from recognition by dsRNA sensors. ADAR1 has dual roles of a negative regulator of ‘‘IFN production’’ in addition to regulating the ‘‘response to IFN
- Human ADAR1 harbors a nuclear localization signal that is composed of a bipartite motif that flanks the third double stranded RNA-binding domain. Interestingly, RNA-binding can mask this nuclear localization signal. Bound double-stranded RNA may also be recognized by exportin 5. Consequently, ADAR1 p110 and p150 can shuttle between the nucleus and cytoplasm, where the RNA binding-status can affect their intracellular localization. ADAR1 p150 is mostly found in the nucleus, while the p110 version is predominantly nuclear
- ADAR2 harbors an N-terminal nuclear localization signal and is exclusively localized to the nucleus. Within the nucleus, ADARs can be found enriched within nucleoli. To this point, no A to I editing of ribosomal RNAs has been detected. However, editing of miRNAs has been detected. It is therefore possible that the nucleolar localization of the ADARs reflects their activities on these small RNAs
- An alternative explanation for the localization of ADARs in nucleoli may be their sequestration to this compartment. This may aid in controlling their intracellular levels with overexpression of substrate RNAs for ADAR2 leading to increased release of ADAR2 from nucleoli

Key Across-Spices Differences in ADAR Biology

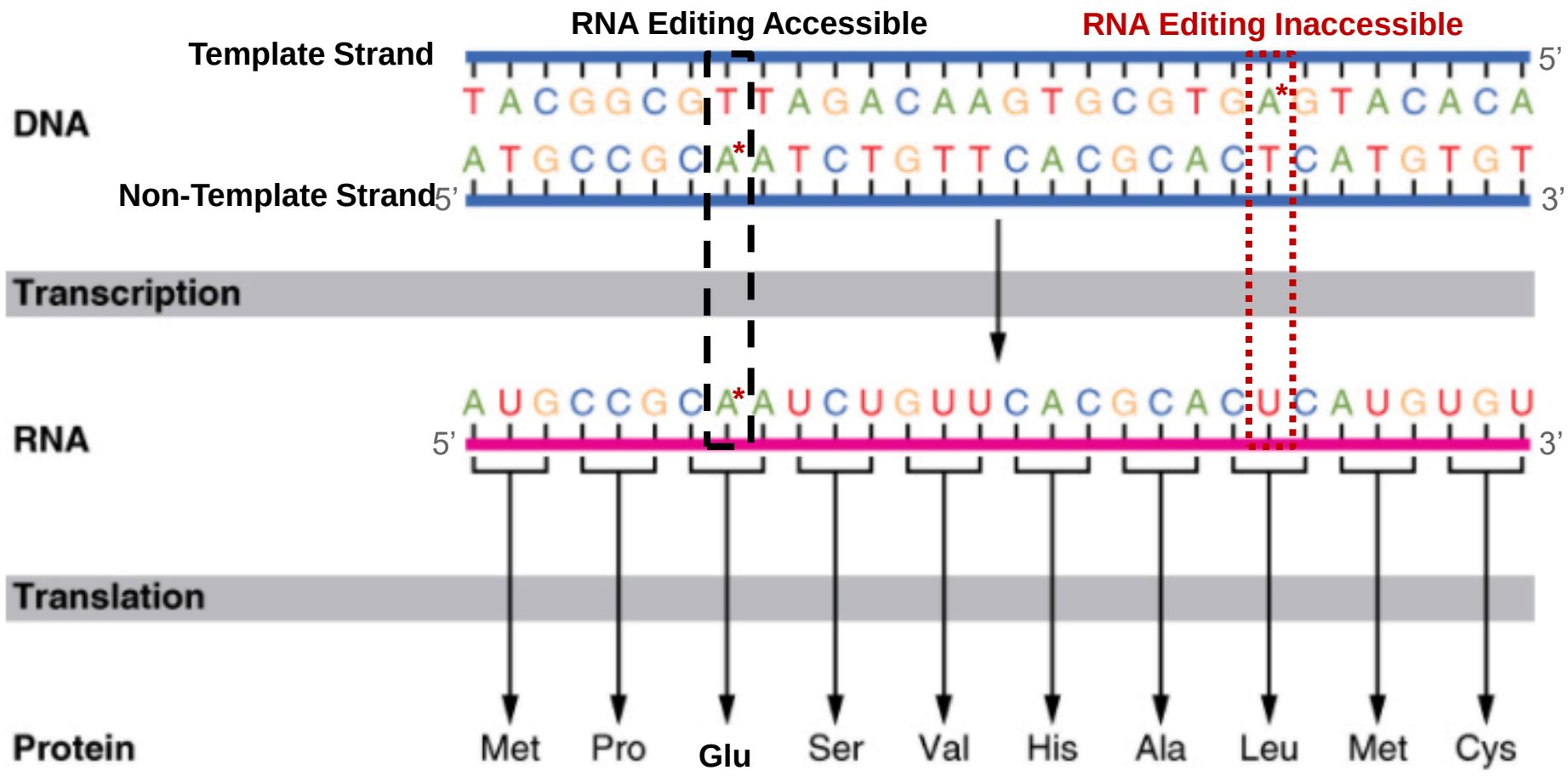
Of the more than 1,000 recoding sites reported in humans, only a few dozen are conserved across mammals— that is, editing occurs at the same location in multiple species. Across-species studies confirm that recoding:

- Is enriched in neural tissues
- Sites are over-represented in transcripts that encode proteins with functions in the nervous system, such as ion channels and neuroreceptor
- Notable examples include the mammalian recoding sites in the serotonin 5-HT2c receptor, in which editing reduces the affinity of the receptor for its G protein31, and the Shaker voltage-dependent potassium channels29 in mammals

In humans, while some A-to-I editing takes place in protein coding regions, the majority of A-to-I editing takes place in primate-specific Alu elements—a class of repetitive SINE (short interspersed elements) retroelements:

- Although the mouse genome contains B1 and B2 SINEs, it lacks Alu elements. Consequently, conserved A-to-I editing sites only minimally overlap between humans and other mammals
- Moreover, A-to-I editing is vastly more prevalent in the human transcriptome than in mice (at least 100-fold)
- In addition, it is not known why Aicardi-Goutières syndrome patients with ADAR1 mutations display neurologic symptoms whereas mice lacking ADAR1 are most severely affected in hematopoietic compartments

A-to-I(G) Editing at the RNA Level IS Equivalent to Addressing G>A Mutations on the Non-template Strand of DNA



Assuming a mutations in the DNA (* = Mutation G-to-A):

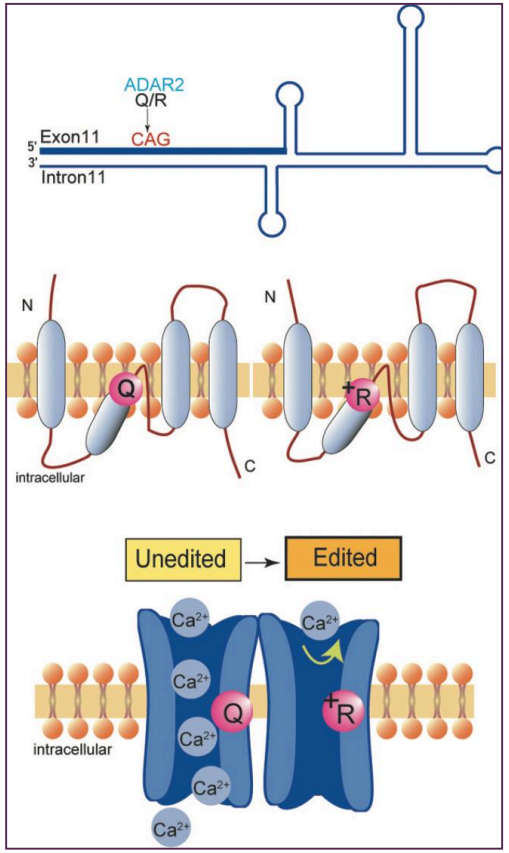
(1) An ABE could act on the A* nucleotide of the DNA non-template (black dashed box) and convert A-to-I(G) restoring the sequence to wild-type

(2) Analogous to bullet 1, RNA editing of A*-to-I(G) would restore the transcript to wild-type without manipulating the genome (black dashed box)

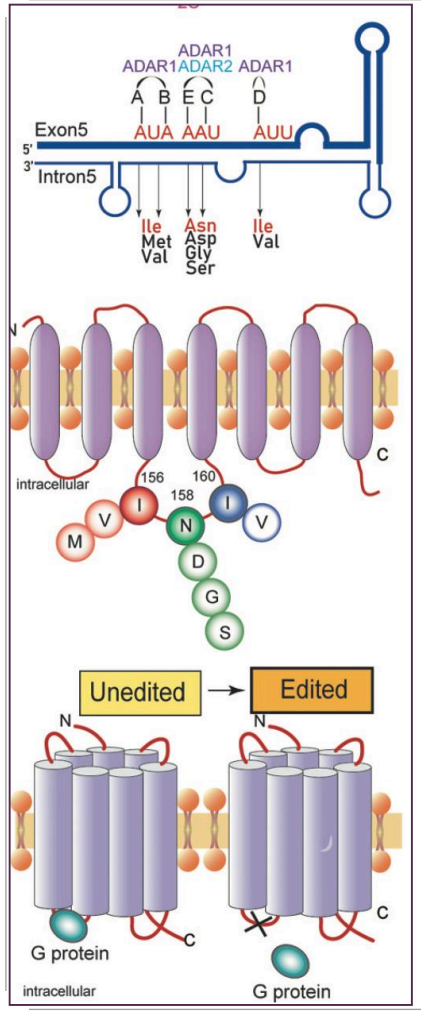
(3) If the G>A mutation occurs on the template strand, RNA editing cannot restore the transcript to the WT sequence (red dotted box)

Source: https://courses.lumenlearning.com/cuny-csi-ap-1/chapter/protein-synthesis/

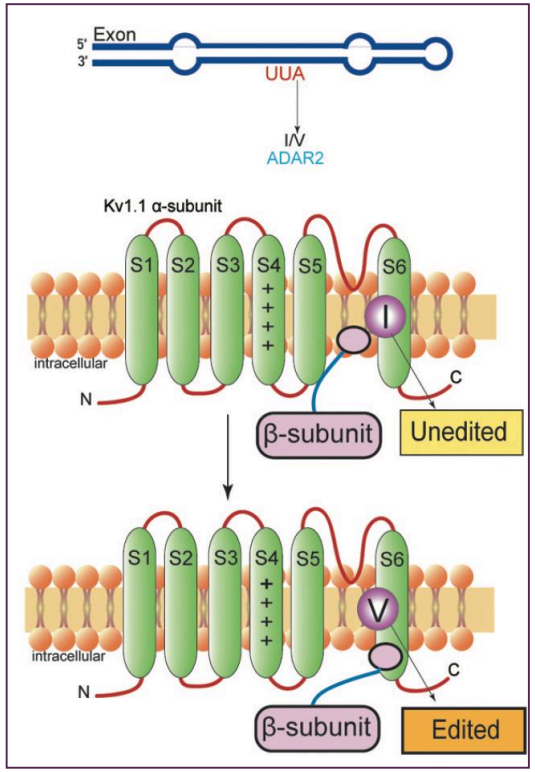
Functional Roles of A-to-I RNA Editing in the Neurotransmitter Receptor and the Ion Channel



The schematic of the GluA2 subunit structure and the functional difference in edited and unedited forms (middle and bottom, respectively). RNA editing alters the glutamate residue (Q) (middle left) at the 607th position to a positively charged arginine residue (R) (middle right), which can dramatically reduce calcium permeability (bottom)



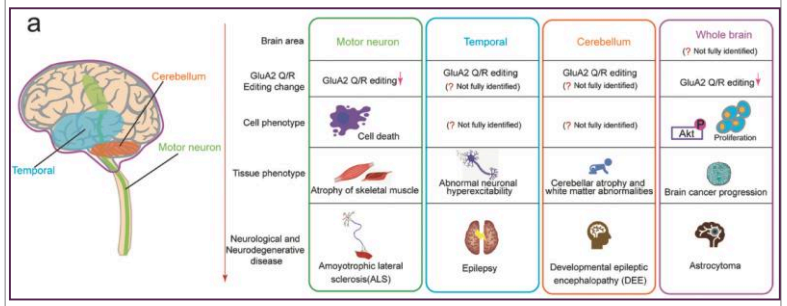
The schematic of 5-HT_{2c} receptor structure the functional difference in edited and unedited forms (middle and bottom, respectively). RNA editing of 5-HT_{2C} receptor converts amino acid isoleucine (I) at 156th position to valine (V), or methionine (M), asparagine (N) at 158th position to aspartic acid (D), serine (S), or glycine (G), and isoleucine (I) at 160th position to valine (V) (middle).
Edited 5-HT2CR isoforms exhibit reduced G protein coupling efficiency (bottom left) in comparison to the unedited isoform (bottom right)



The schematic of Kv1.1 channel structure and the functional difference in edited and unedited forms (middle and bottom, respectively).
The transmembrane voltage sensor (S4), the K^+ ion selectivity filter (S5–S6) are indicated in the middle and bottom figure. Editing of Kv1.1 changes the isoleucine (I) (middle) to valine (V) (bottom). which reduces the affinity for the binding of Kv β1.1 and enhances recovery from inactivation

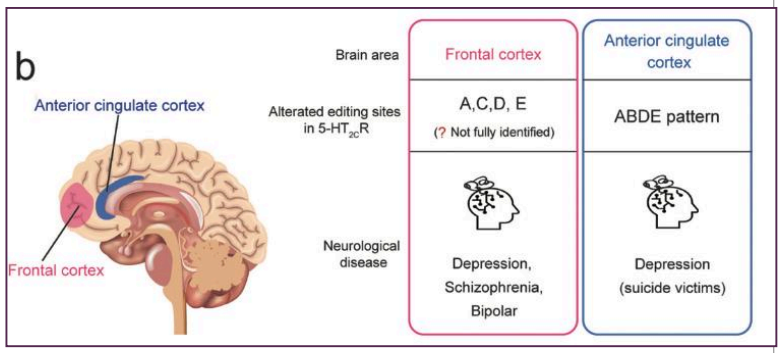
Source: https://pubmed.ncbi.nlm.nih.gov/33393416/

Neurological or Neurodegenerative Diseases Associated with Dysregulated A-to-I RNA Editing

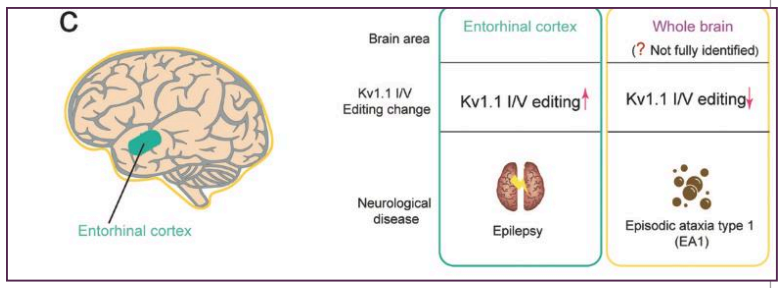


ADAR2 deficient mice exhibit postnatal death due to epileptic seizures caused by under-editing at GluA2 Q/R site. A recent study has identified the *de novo* heterozygous variants in GRIA2 among individuals with intellectual disability and neurodevelopmental abnormalities.

One of the most profound variants, Q607E, occurred at the Q/R site in an individual with a severe DEE. These findings suggest that the epilepsy phenotypes observed in DEE patients arising from bi-allelic ADAR2 variants may be attributable in part to under-editing of the GluA2 Q/R site



The accumulating evidence suggests that alteration of editing patterns of 5-HT2C R mRNA may be associated with several psychiatric disorders, including anxiety, depression, bipolar disorder, and schizophrenia



The transcript encoding Kv1.1 is subjected to the conversion of the codon ATT to ITT through RNA editing in a region encoding the sixth transmembrane domain, resulting in a change of isoleucine to valine (I/V).

A four-fold increase in the Kv1.1 editing level was observed in the entorhinal cortex of chronic epileptic animals, and a reduction in the potency of the Kv open channel blocker 4-aminopyridine that induces seizures was observed, suggesting that the addition of Kv1.1 transcript editing helped suppress seizures

Source: https://pubmed.ncbi.nlm.nih.gov/33393416/

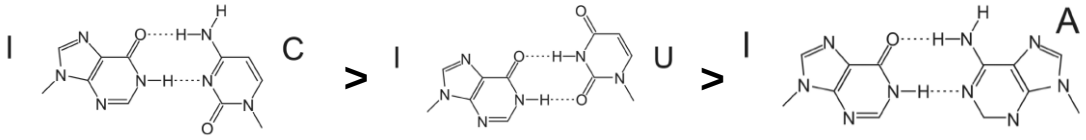
Risk: Non-Human Data Suggest *Inosine Read and Guanine* Assumption May Have Exceptions

"…while inosine is primarily interpreted as guanosine it can also be decoded as adenosine, and rarely even as uracil."

Key Message: There is innate flexibility in base pairing with inosine that argues for a more complex picture of how cellular machinery reads the presence of I in the RNA. Experimental work suggests qualitatively I is not always read as G. However, initial data (right) were generated using rabbit reticulocyte lysate. Strikingly, IAC (putatively read as GAC) was read as AAC encoding asparagine as opposed to aspartic acid

- Position 34 of 8 cytosolic tRNAs include an inosine at the 5' end (aka the wobble position) of the anticodon and can pair with C, A or U
- Transiently, non-Watson/Crick base-pairing is tolerated during codon/anticodon pairing outside of the wobble position
- DNA-helices are not destabilized by inosine paring with C, U, or A

Stability



inosine codon		amino acid		decoding	summed intensity
IAA		Glu	→	GAA	959.7 x 10^7
		Lys	→	AAA	16.9 x 10^7
ICC		Ala	→	GCC	1014.5 x 10^7
		Thr	→	ACC	4.4 x 10^7
~30% IAC		Asp	→	GAC	818.3 x 10^7
		Asn	→	AAC	245.0 x 10^7
ICA		Ala	→	GCA	403.5 x 10^7
		Thr	→	ACA	6.6 x 10^7
		Ser	→	UCA	3.4 x 10^7
~9% IAI		Glu	→	GA*	79.1 x 10^7
		Lys	→	AA*	6.9 x 10^7
ICI		Ala	→	GC**	165.0 x 10^7
		Ser	→	UC**	1.8 x 10^7
IIA		Gly	→	GGA	483.2 x 10^7
		Arg	→	AGA	9.6 x 10^7

* 3rd pos. G or A
** 3rd pos. G, A or U

Source: https://pubmed.ncbi.nlm.nih.gov/30462291/

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ADAR Platforms Overview

Endogenous ADAR-Based Editing—cadRNA Increases Editing Efficiency and Durability

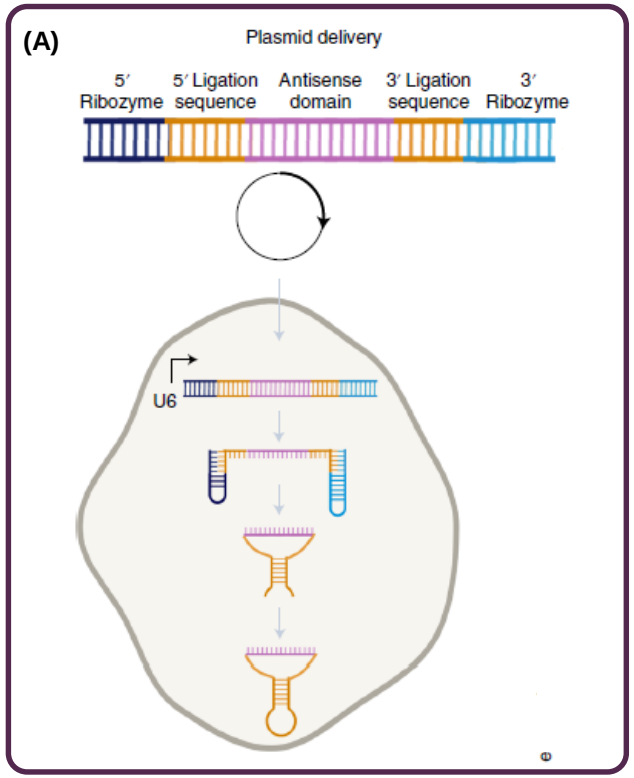
Plasmid Delivery and Post-Expression Processing Yields cadRNA

Key Message: (1) Persistence of adRNA appears to be a major modulating factor in endogenous ADAR-based editing; (2) Circular RNA increases target RNA editing efficiency and durability in cellular systems; (3) cadRNA localizes to both the cytoplasm and the nucleus

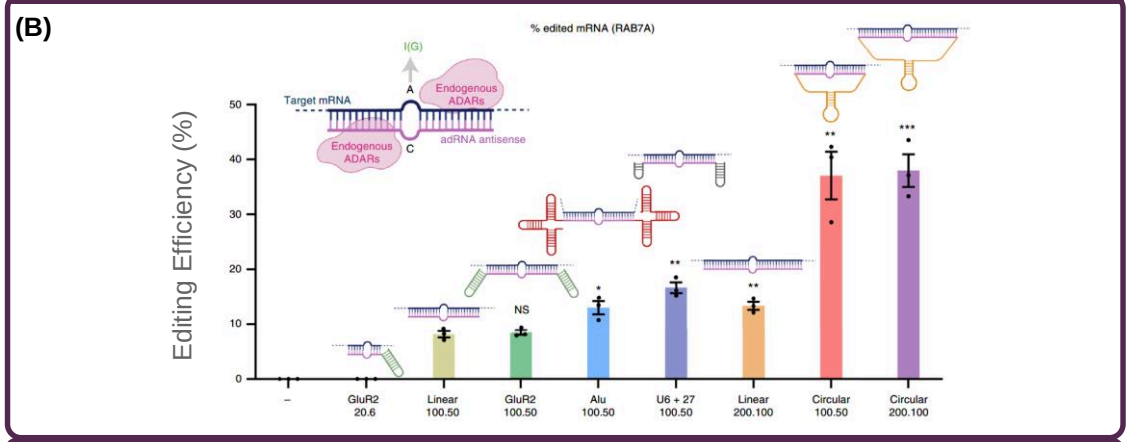
(A) The cadRNA construct can be delivered to cells using a plasmid. Following expression of the pre-cadRNA vector, enzymatic processing via (1) twister ribozymes and (2) ubiquitous endogenous RNA ligase RtcB yields cRNA

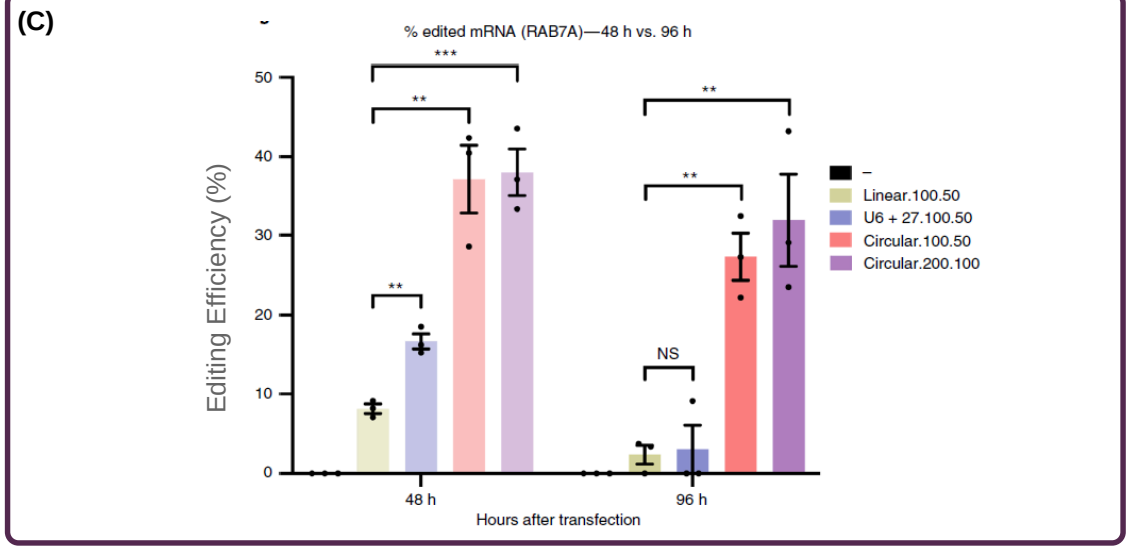
(B) Circularization leads to greater stability relative to linear adRNA

(C) The durability of cadRNA is greater than linear adRNA constructs



cadRNA's Superior RNA Editing In HEK293FT Cells Rel. Linear adRNA

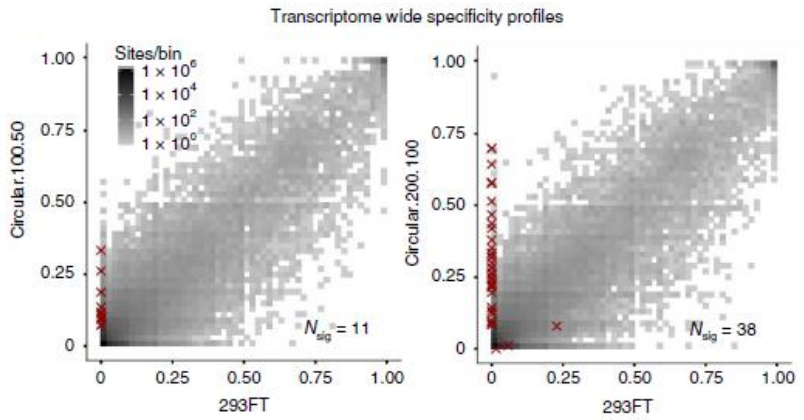




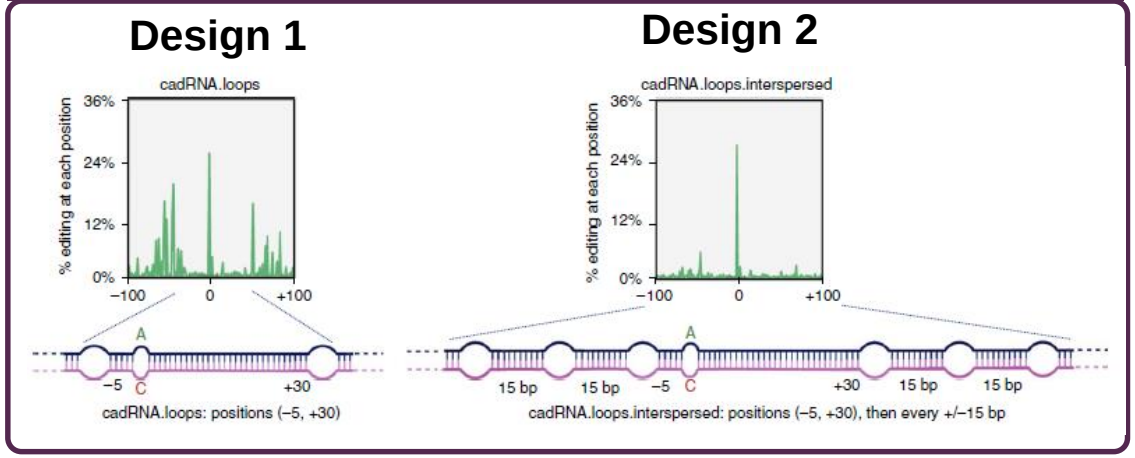
Also see slides on EdiGene that cover circular RNA-based RNA editing
Source: https://pubmed.ncbi.nlm.nih.gov/35145312/

CadRNA Off-Target Editing (11-38 Events) Reduced Relative to ADAR Over Expression (~10^3)

Transcriptome Profile Shows Low Frequency of Off-Target Editing



Bystander Editing Reduction via Antisense RNA G:A Bulges



The occurrence of off-target editing was evaluated for two cadRNA constructs targeting the RAB7A transcript. Constructs were delivered via the plasmid system as described on the previous slide.

(1) The number of significant off-target events was 11 for construct Circular.100.50 and 38 for Circular.200.100;

(2) Off-target editing frequency ranged from ~30% to ~70%

Two cadRNA constructs were examined for bystander editing at the RAB7A transcript.

(1) The Design 1 construct showed an on-target A>I(G) editing frequency of ~24%. However, as depicted above, a number of bystander edits were observed (signals +/- relative to 0)

(2) Design 2, incorporating G:A bulges at bystander sights appreciably reduced off-target editing

Source: https://pubmed.ncbi.nlm.nih.gov/35145312/

Mouse *In Vivo* RNA Editing Example Demonstrates Feasibility—Therapeutically Limited

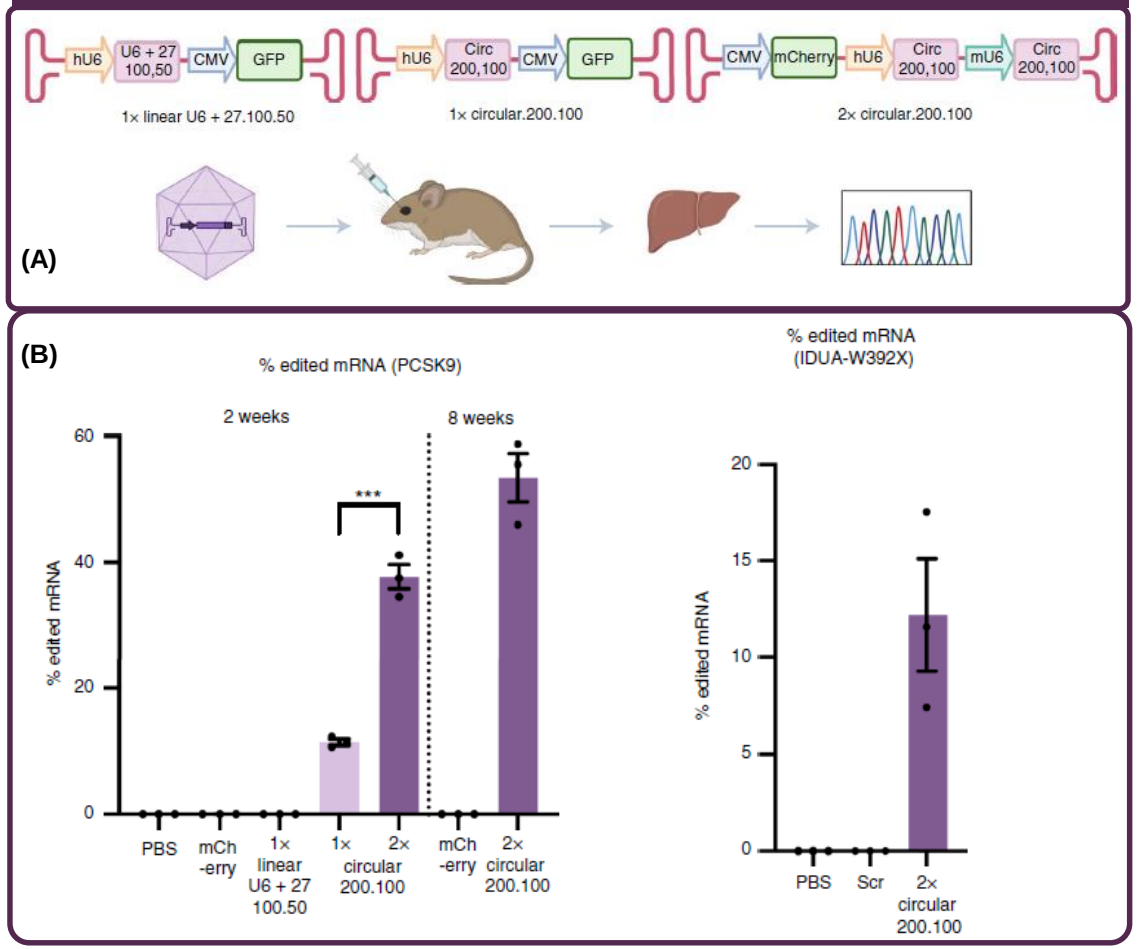
RNA Editing Fall Short of VERVE-101 for Relevance and Efficiency

Key Message: (1) *In vivo* editing of the 3'-UTR of the mouse PCSK9 transcript was achieved ~55% at 8 weeks; (2) 3'-UTR does not appear to be a therapeutically-relevant target for PCSK9; (3) the highest level of *in vivo* PCSK9 transcript editing falls short of VERVE-101 (VERV) data reporting ~70-80% therapeutic editing in NHPs; (4) MPS I mouse model example shows ~12% editing which resulted in ~30% reduction in GAGs in the liver:

(A) CadRNAs were delivered for *in vivo* RNA editing *via* AAV and vectors encoding 2X circular 200.100 adRNA appeared to result in greater editing

(B) Two mouse models were evaluated for transcriptome editing. Both showed Editing ≤ 55%

Mouse Models for PCSK9 and MPS I



Sources: https://pubmed.ncbi.nlm.nih.gov/35145312/, nature.com/articles/s41586-021-03534-y.epdf?sharing_token=HsonYNLZxtKY14BoswRIRtRgN0jAjWel9jnR3ZoTv0M6Hd6FaG8LKZpFk4zRG3bRk_k-yu6JL-SBSnxGMXRwXflxZsV5lzyijNLy4qu_cSv4Njqsxu4TZYFLKqINGhvzwDQdUC2kvLRcc5A7mJcY0h8UigbTDJ4CDJRlu00lw70%3D

Engineered ADAR2 Domains Can Overcome Refractory 5'-GAN-3' RNA Editing

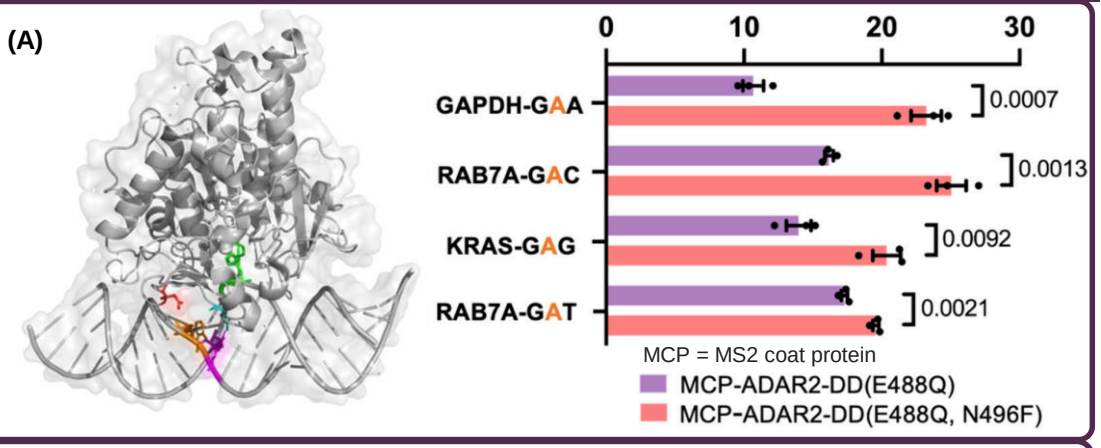
Editing 5'-GAN-3' and Split Approach to Enhance Specificity

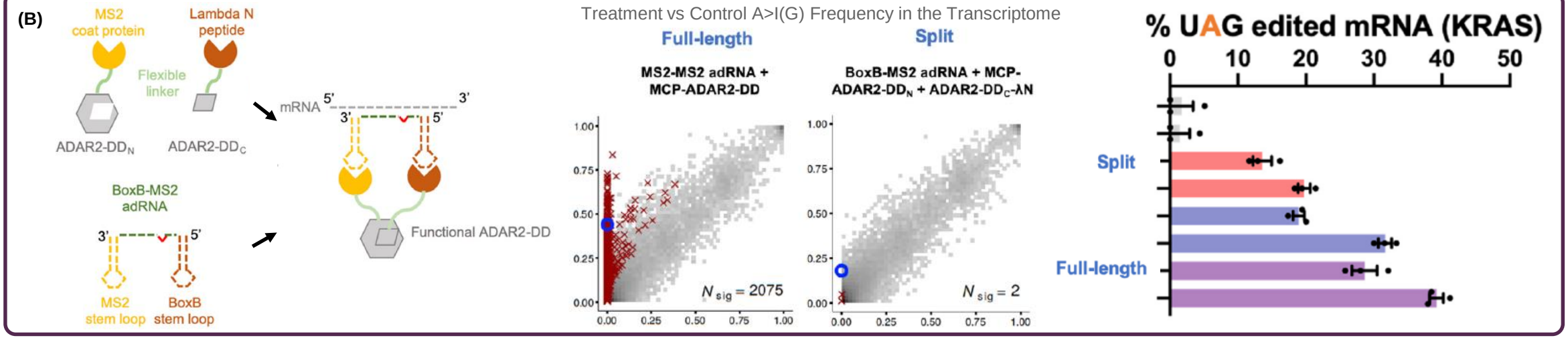
Key Message: Analogous to base editors for genome editing, engineered ADAR2 and adRNA can be transfected into cells. The adRNA pairs with the target RNA and recruits the engineered ADAR2 to install an A>I(G) edit:

(A) Optimization has identified the MCP-ADAR2-DD(E488Q, N496F) construct able to edit at refractory 5'-GAN-3' RNA motifs

(B) Split ADAR2-DD approach decreases off-target editing relative to full-length construct albeit at a cost to editing efficiency

E488Q and E488Q/N496F Mutant Can Edit Refractory 5'-GAN-3'





Source: https://pubmed.ncbi.nlm.nih.gov/35044296/

ADAR2 Derivatives Fits Into an AAV System and split-RESCUE Accesses C>U RNA Mutations

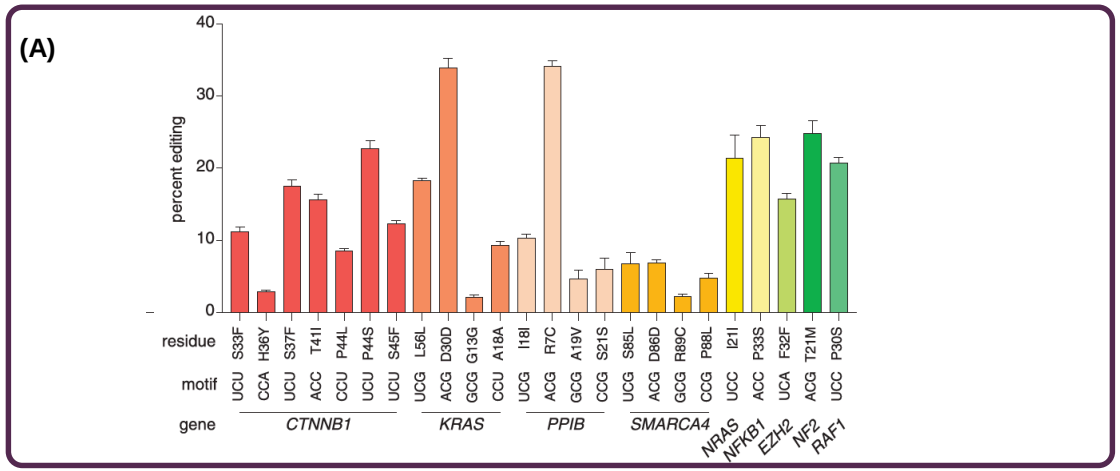
Zhang's Vision Carried Forward with split-RESCUE

Key Message: The split- ADAR2 system (including promoters) is about ~3500 bp in size, which would support packaging into AAV. Additionally, a split-RESCUE platform was created that can make C>U mutations in RNA, albeit at low editing efficiencies:

(A) RNA Editing for Specific C-to-U Exchange (RESCUE) was originally created by the Feng Zhang lab

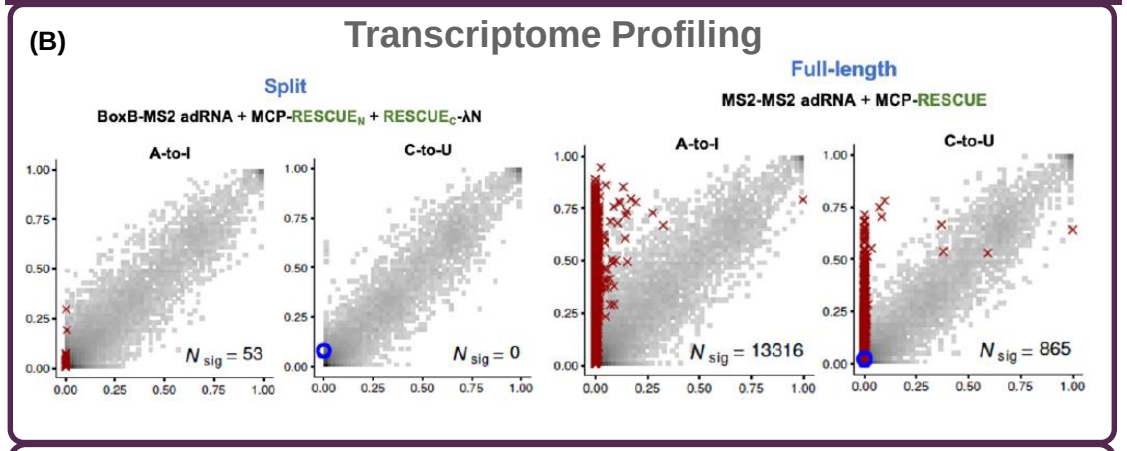
(B) Split RESCUE shows lower off-target editing than the full-length construct

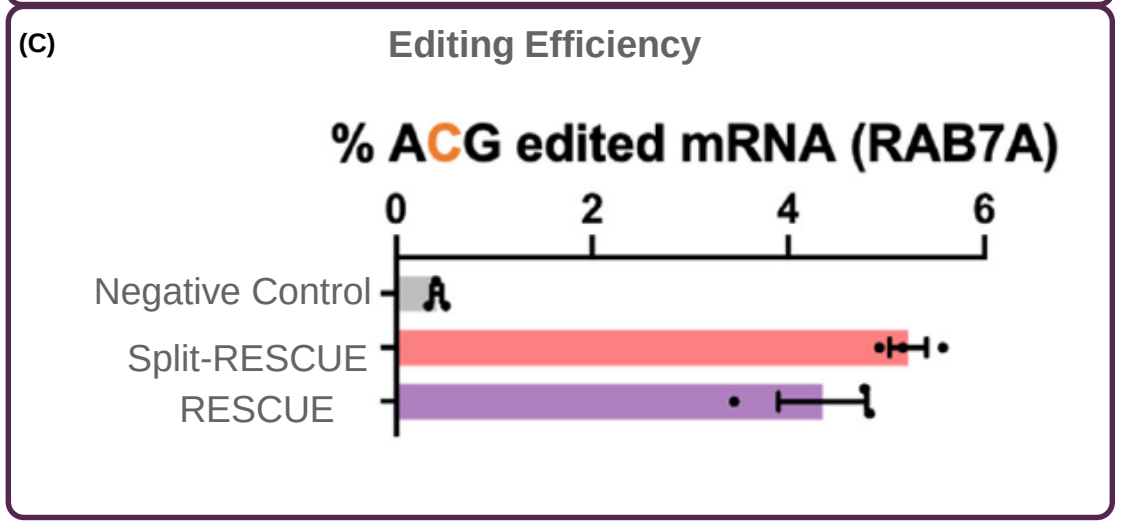
(C) Overall editing efficiency was low for both the full-length and split-RESCUE platforms



Sources: https://pubmed.ncbi.nlm.nih.gov/35044296/, https://pubmed.ncbi.nlm.nih.gov/31296651/

Split Platform for C-to-U— Minimal Off-Target Editing





Evolving Application of Cas13-ADAR: A-to-I(G), C-to-U, and Shrinking in Size

Key Parameters for Cas13-based RNA Editing Have Been Defined

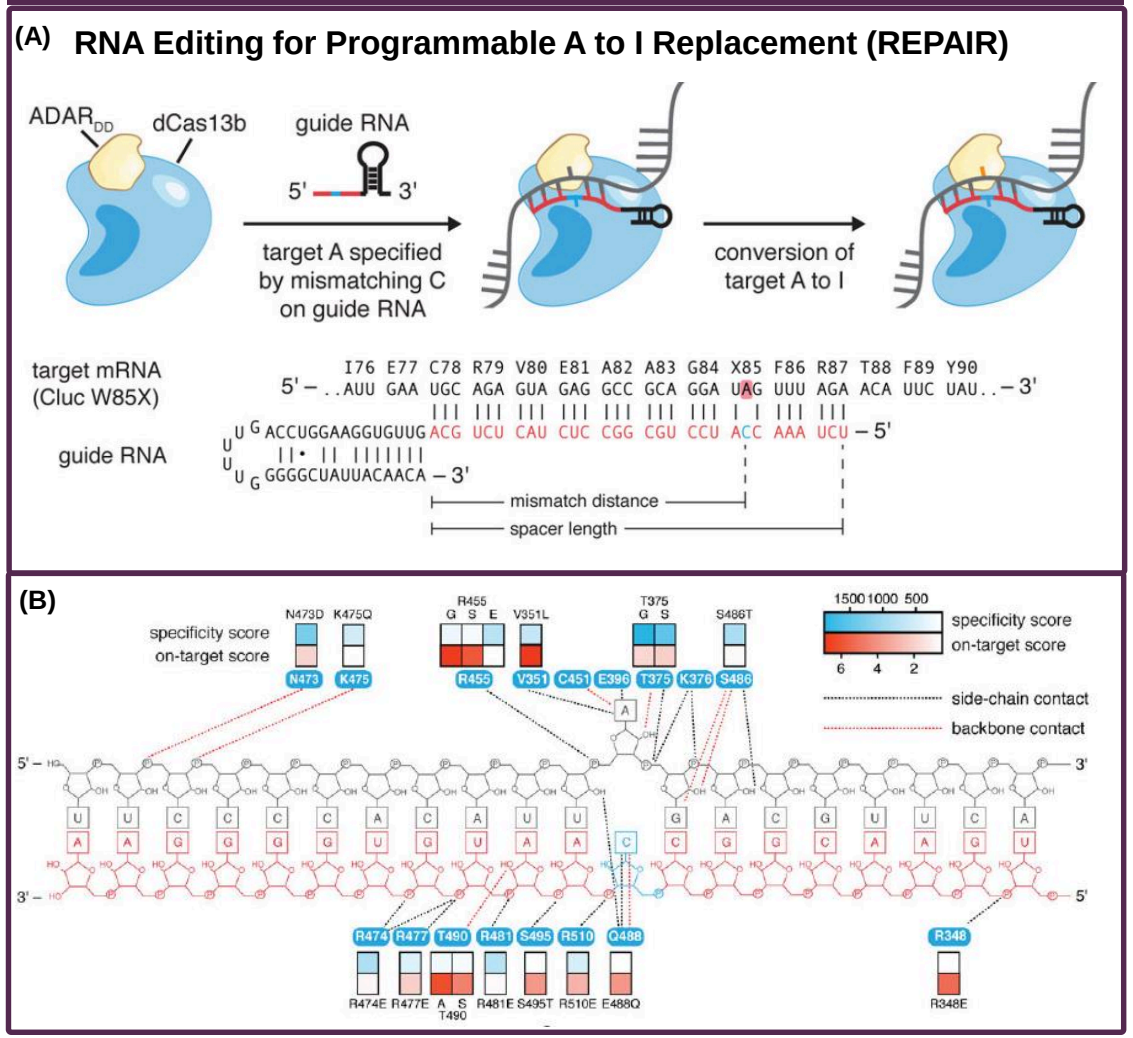
Key Message: Cas13 fused to ADAR for (1) gRNA-facilitated localization to a target transcript followed by (2) deamination of an adenine base for A-to-I(G) transformation emerged from the laboratory of Feng Zhang (Broad Institute). Cas13 encoded in the gnomes of *Prevotella sp.* (REPAIR) and *Riemerella anatipestifer* (RESCUE) has demonstrated the most efficient mRNA knock down activity amongst the Cas13 family:

(A) Ligation of hyperactivated ADAR1DD(E1008Q) or ADAR2DD(E488Q) yields the REPAIR platform able to localize to a traget transcript *via* a complement guide RNA; a mismatch between the target A and guide C enhances editing efficiency

(B) Work from Zhang's Laboratory characterized influential contacts between key ADAR2 deaminase residues and the double stranded RNA. We see this work as foundational in the Cas13-based RNA editing field and will greatly accelerate the further development of these platforms

Sources: https://pubmed.ncbi.nlm.nih.gov/29070703/, https://pubmed.ncbi.nlm.nih.gov/31296651/, https://pubmed.ncbi.nlm.nih.gov/34462587/

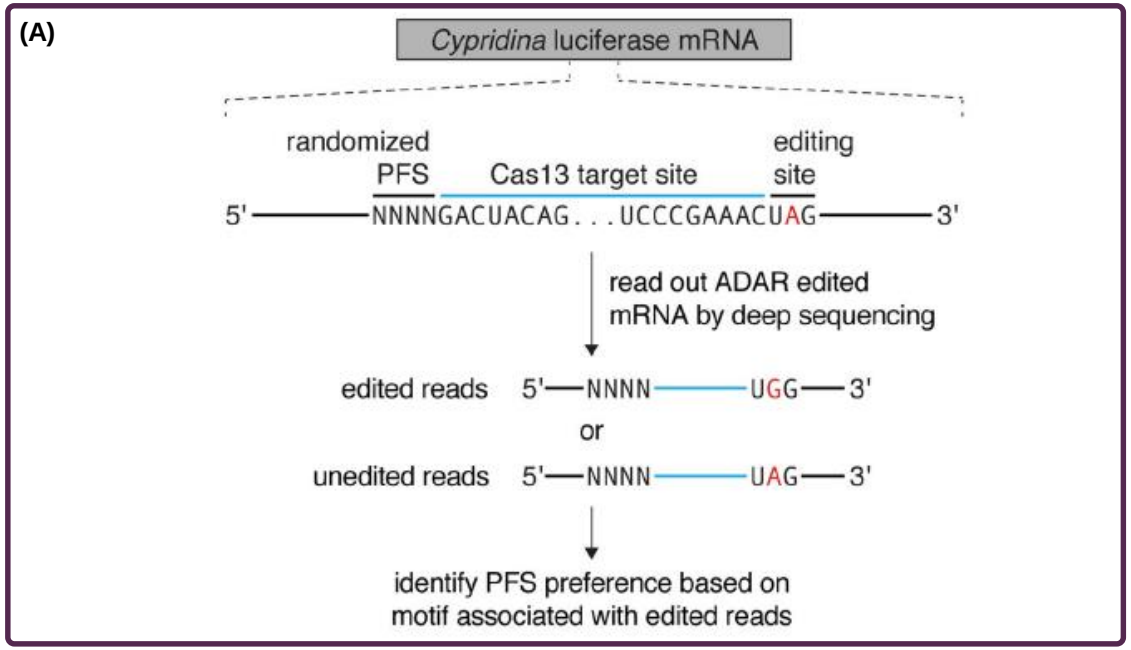
dCas13b Fused to the Deaminase Domain of ADAR2$_{DD}$



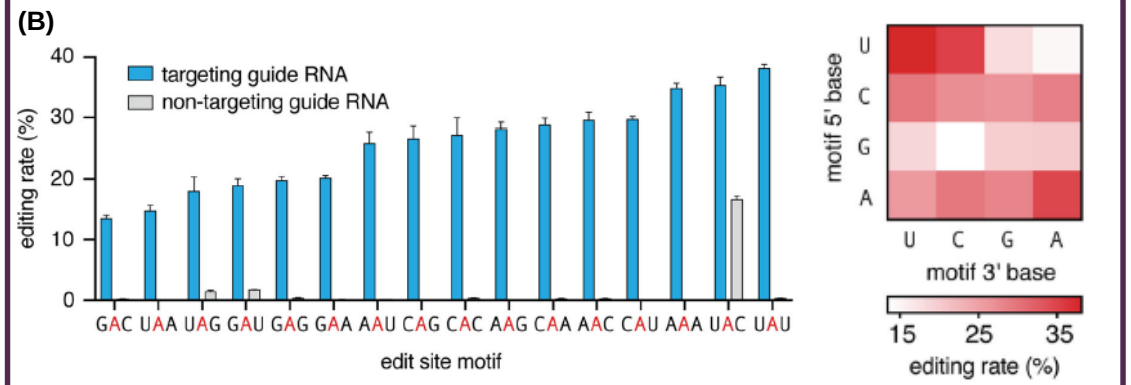
ADAR Domain Fused to Cas13b Yields REPAIR—Optimization Markedly Lowers Off-Target Edits

No PFS-Dependency; A Unique Attribute Relative to CRISPR's PAM

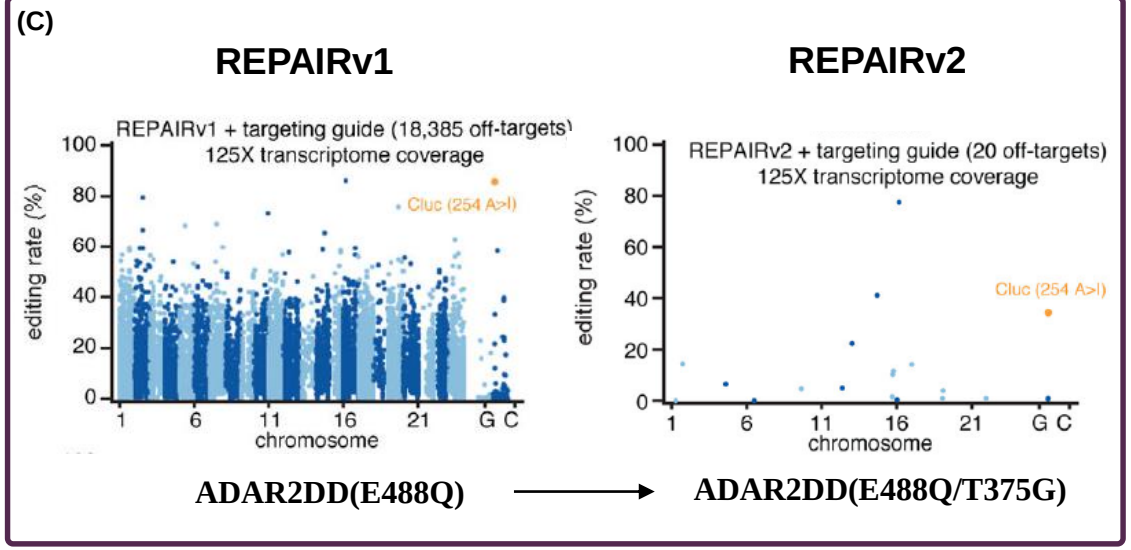
Key Message: (A) The dependency of RNA Editing for Programmable A-to-I (REPAIR) on protospacer flanking site (PFS) motif was evaluated in a plasmid library for the *Cluc* transcript; (B) Across various target site motifs, the editing rate was determined to be approximately 13% to 38% *in vitro*; (C) The high level of off-target editing observed in the transcriptome by the REPAIRv1 construct (~18k with off-target editing rates ≤ 90%) were addressed by using REPAIRv2



REPAIRv1 Editing Efficiency ~40% and Flexible Editing Site Motif



REPAIRv2 Dramatic Improvement in Transcriptome Off-Target Editing

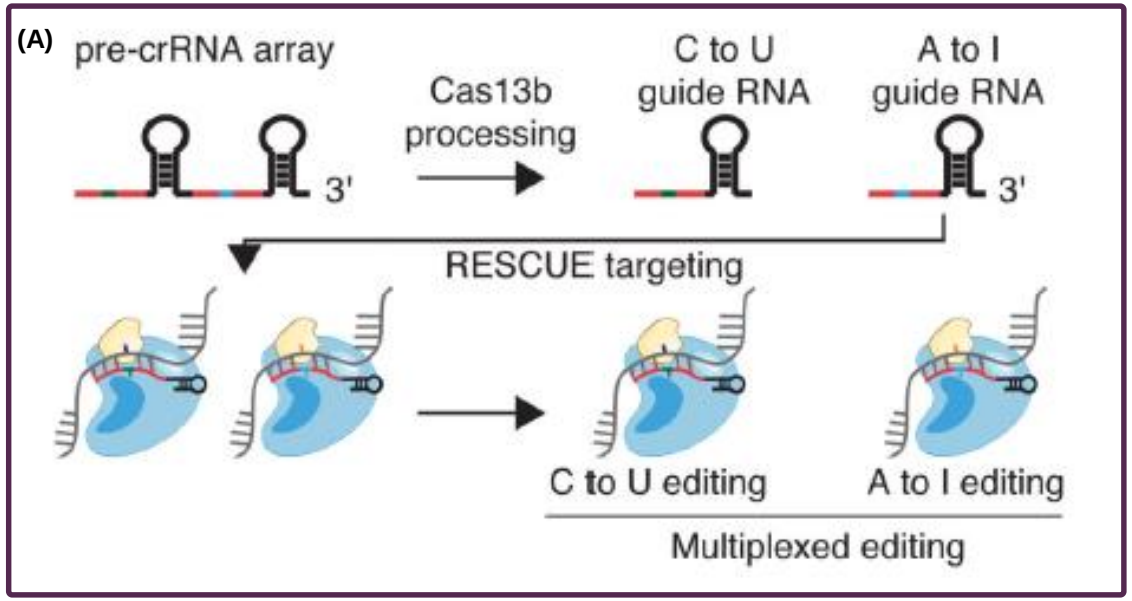


Source: https://pubmed.ncbi.nlm.nih.gov/29070703/

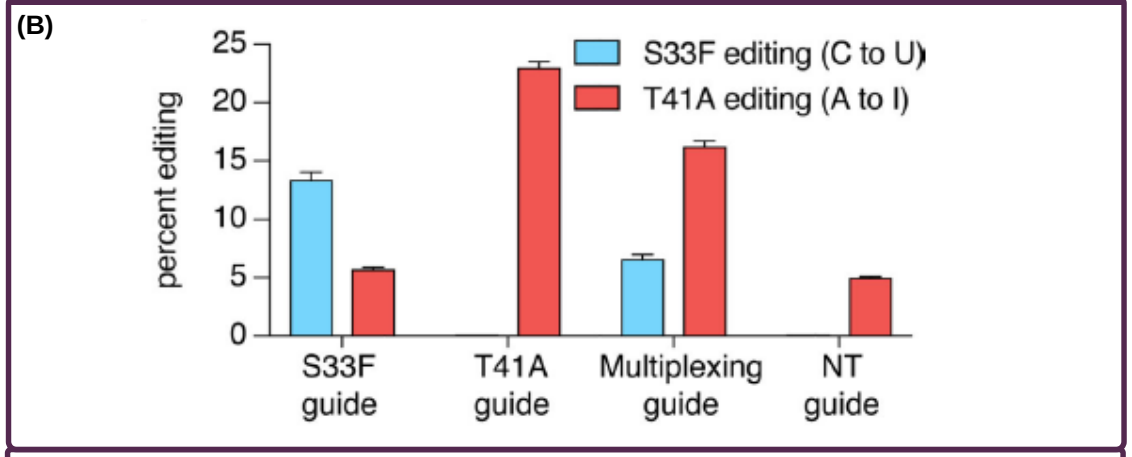
RESCUE a Two-Prong Platform that Can Enact C-to-U and A-to-I in RNA

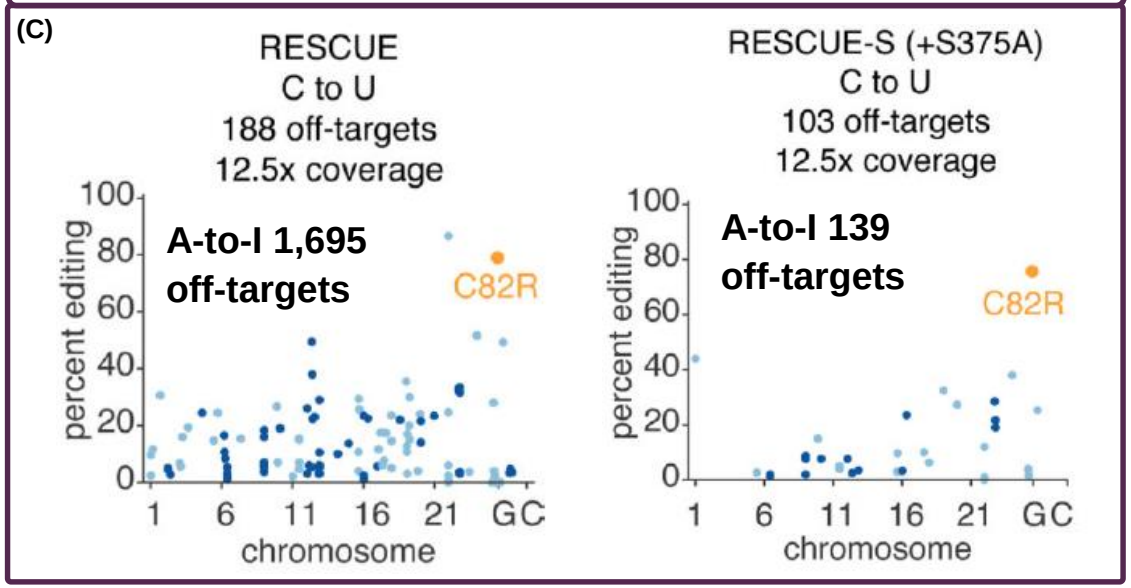
Retention of A-to-I Functionality Support Multiplex Editing

Key Message: (A) Using a pre-crRNA array, Cas13b can yield two individual guide RNAs (facilitating C-to-U and A-to-I) to direct multiplex editing to two sites in the transcriptome; (B) multiplex editing on the *CTNNB1* transcript in HEK293FT cells shows 5% to 15% editing with 5% editing observed using a Non-targeting guide strand; (C) off-target editing rates were high for RESCUE (r16) but the S375A mutation to yield RESCUE-S appreciably lowers off-target editing with minimal alteration to on-target editing (C82R)



Approximately 5% to 15% Multiplex Editing Rates





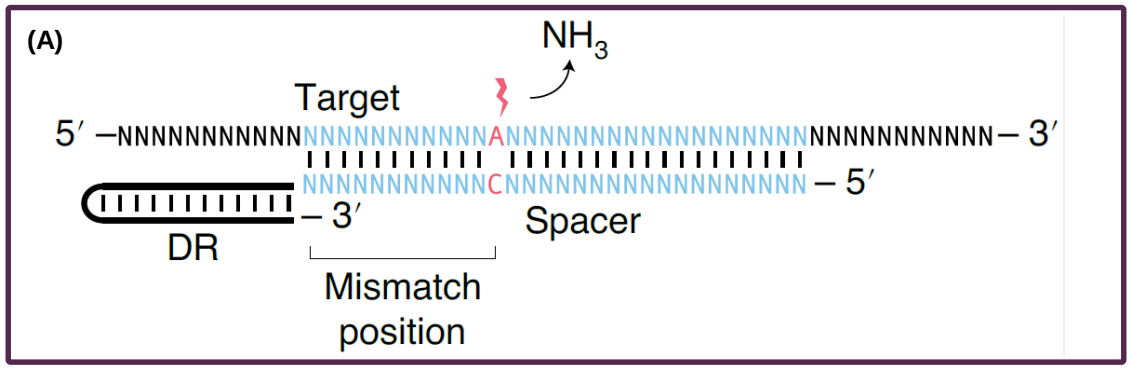
Source: https://pubmed.ncbi.nlm.nih.gov/31296651/

REPAIR.t1 (A-to-I) and RESCUE.t1 (C-to-U) ~20% Size Reduction Supports AAV2 Delivery

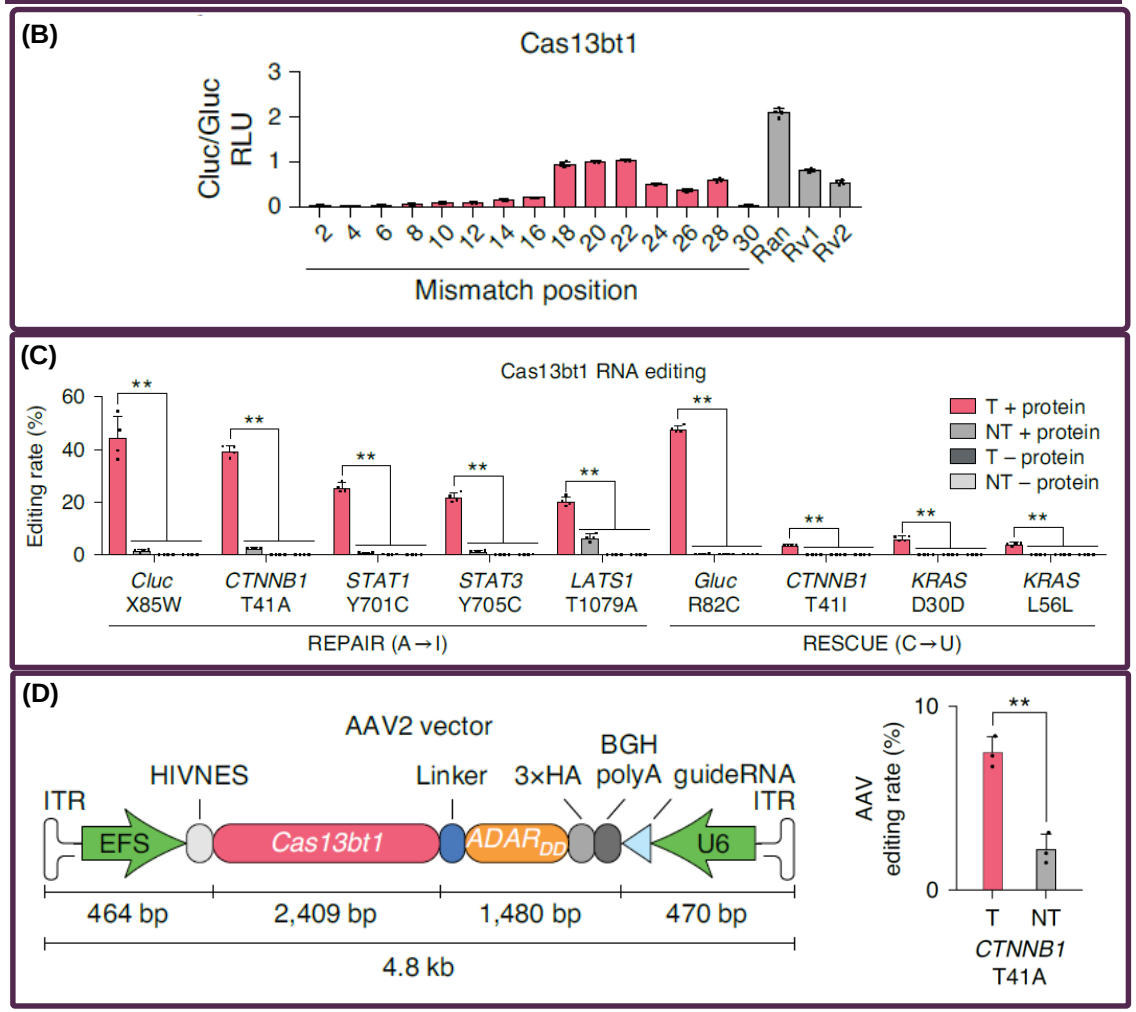
Optimal Positioning for the A:C Mismatch for A-to-I(G) Editing

Key Message: Cas13bt members are reduced in size (775-804 aa) relative to BzoCas13b (1,224 amino acids (aa)) facilitating the construction of smaller REPAIR and RESCUE platforms

(A and B) The positioning of the mismatched (A-C) for editing efficiency optimization was determined to be 18-20 nt from the 3' end (first pairing of target and spacer) of the guide sequence; (C) editing efficiency reaches at most ~40% for REPAIR.t1 and RESCUE.t1; (D) AAV2 vector has been designed that integrates REPAIR.t1 for viral delivery, albeit resulting *in vitro* editing is < 10% (study was conducted in HEK293T cells and non-targeting guide (NT))



AAV2 Delivery Possible – Editing Rate < 10%



Source: https://pubmed.ncbi.nlm.nih.gov/34462587/

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RNA Editing Companies

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Wave Life Sciences (WVE) – AIMers for RNA Editing

Executive Summary for Wave RNA Editing Programs

Wave takes a synthetic chemistry approach for the development of oligo AIMers for endogenous ADAR-based RNA editing:

1. Diseases where the technology has been indicated as applicable include alpha-1 antitrypsin and Rett syndrome
2. The Rett approach uses an unnatural compensatory R168W mutation to restore function of MeCP2 with transient transcriptome editing potentially enabling for this exploratory approach

Wave's Rett program shows MeCP2 restoration *in vitro via* compensatory (R168W) mutation:

1. Wave's Rett AIMer is able to yield a compensatory R168W MeCP2 mutant putatively a path forward to address the c.502C>T, p.(Arg168*) nonsense mutation
2. The frequency of this mutation has been estimated at ~8% in the Rett population
3. Biochemical data demonstrate recapitulation MeCP2 signaling pathway

AIMer for the treatment of A1ATD shows ~40-75% editing efficiency:

1. Some off-target editing was observed, albeit at a lower frequency than target PiZ transcript editing frequency
2. At 40% to 70% on-target editing and with clinically-relevant serum AAT concentrations in sight (*in vivo* mouse model), improvements in *in vivo* editing efficiency positions WVE for a reasonable clinical outlook
3. Preclinical data from NTLA and BEAM suggest (1) their A1ATD programs offer complementary genomic editing approaches relative to WVE' RNA editing strategy and (2) WVE's AIMer approach may be a hedge for unforeseen issues encountered with platforms such as BEAM-301 that target A1ATD repair *via* genome editing

AIMers can be implemented to disrupt protein-protein interactions:

1. KEAP1 or Nrf2 example provide proof of concept preclinical data to demonstrate RNA editing via AIMers can be used to facilitate the disruption of protein-protein-interactions

Wave Demonstrates AIMer Potency Optimization and Shows 20-70% mRNA Editing

AIMer Enabled to Enact RNA Editing

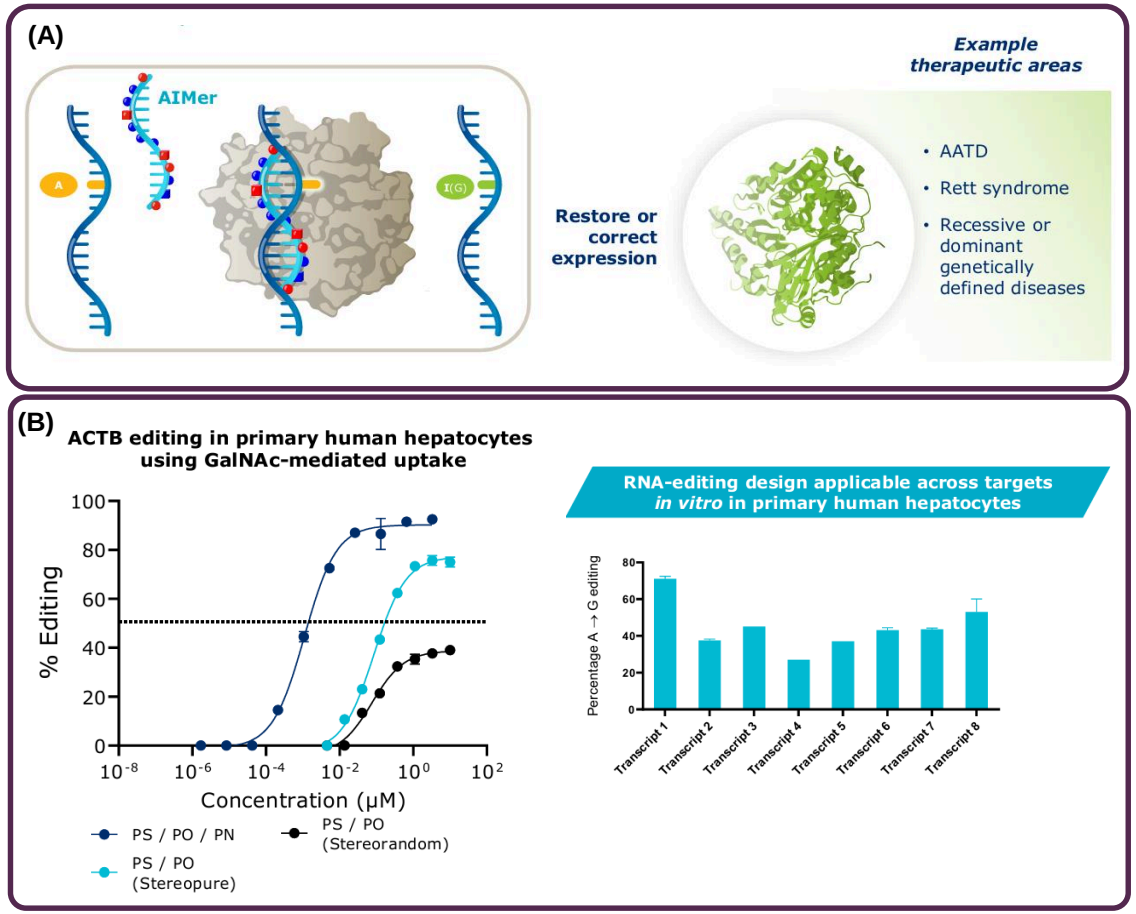
Key Message: The AIMer platform developed by Wave leverages synthetic RNA molecules to base pair with a target mRNA strand. The binding event results in a tertiary dsRNA structural motif recognized by the adenosine deaminases acting on RNA (ADAR) enzyme. ADAR is able to catalyze specific A to I conversions. Leveraging the AIMer platform Wave has achieved 25-70% mRNA editing efficiency

(A) Diagram showing the formation of dsRNA from the AIMer and target mRNA and complexation with ADAR. This approach is applicable over a range of diseases as listed in the figure

(B) The *ACTB* gene transcript can be targeted with the ADAR approach. Wave has developed synthetic chemistry methods to control the AIMer sequence, ribose chemistry, and phosphoramidate (PN) or phosphorothioate (PS) stereo chemistry. Wave claims that control over oligo chemistry can be used to increase the potency of an AIMer series

Source: WVE Corporate Presentation, March 3, 2022 (https://ir.wavelifesciences.com/static-files/4e03e4de-0db7-45c6-8673-b855de4ca6b0)

Mechanism and Potency of AIMers



Wave's Rett Program Address Nonsense Coding by Leverage a Compensatory Mutation

Wave's MeCP2 Therapy Could Address 8% of the Rett Population

Key Message: Wave is developing a Rett AIMer able to yield a compensatory R168W MeCP2 mutant putatively a path forward to address the c.502C>T, p.(Arg168*) nonsense mutation. The frequency of this mutation has been estimated at 8% in the Rett population. Biochemical data demonstrate recapitulation MeCP2 signaling pathway:

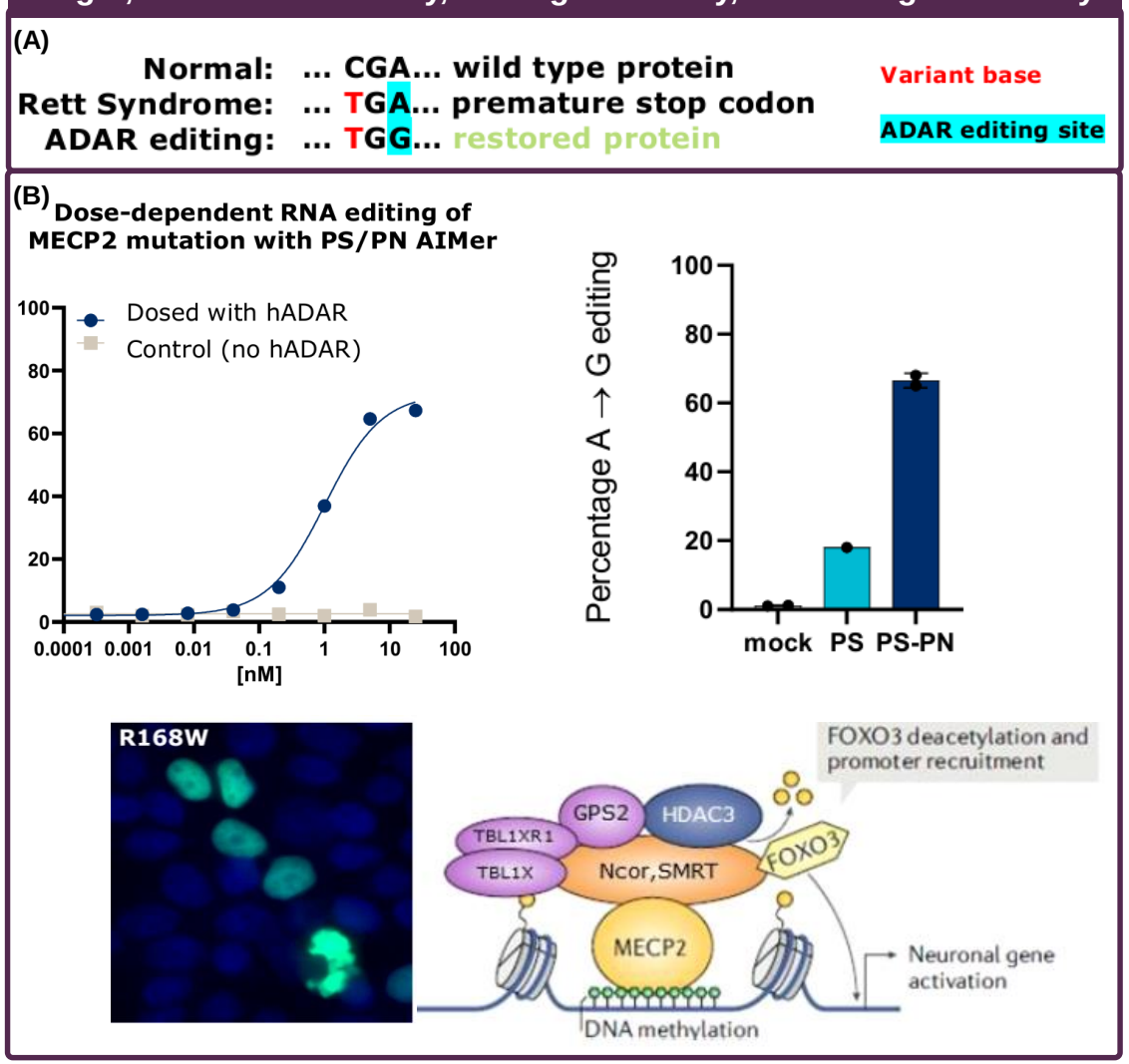
(A) Targeting the transcript derived from the **502C>T** mutation:

- Mutations in *MeCP2* are found in more than 95% of classic RTT
- **Guggenheim's analysis using RettBASE**: (1) Individuals with Rett syndrome harboring only the 502C>T mutation represent ~8% of the Rett patients (2) and patients with 502C>T and additional mutations in *MeCP2* represent < 0.2% of the Rett population
- **Guggenheim's analysis** suggests the top eight missense and nonsense mutations account for 46% of all mutations observed in classical Rett syndrome and originate from C>T mutations
- Mutations affecting the NLS of MeCP2 or early truncating mutations tend to cause the most severe symptoms

(B) Preclinical data demonstrating: (1) a ~2 nM IC_{50} for the *MeCP2* transcript-targeting AIMer, (2) ~70% editing efficiency (3) appropriate cellular localization and evidence for appropriate protein colocalization (NCoR1, TBLR1, and HDAC3) suggesting appropriate functionality

Sources: Wave Life Sciences Presentation March 3, 2022 (https://ir.wavelifesciences.com/static-files/4e03e4de-0db7-45c6-8673-b855de4ca6b0), Wave Life Sciences Presentation September 28, 2021 (https://ir.wavelifesciences.com/static-files/4b998f29-712e-4077-8afd-37d86eb2a9ec), https://www.nature.com/articles/s41597-020-00794-7.pdf

Target, Preclinical Potency, Editing Efficiency, and Biological Activity

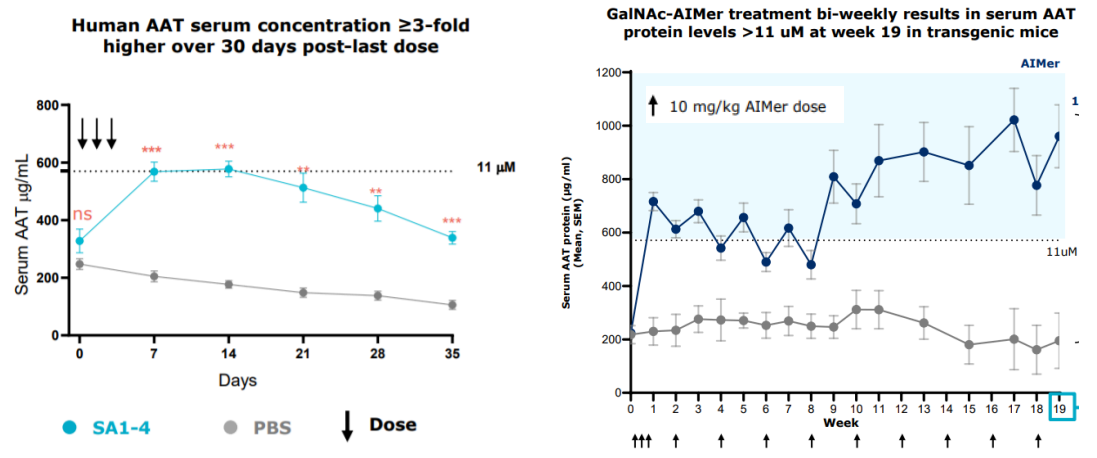


AAT Program Showing ~60% Editing—Q2W Dosing Boosts Serum A1AT Concentrations

huADAR1 / huSERPINA1-Pi*Z Mouse Model no Pathology Rescue

Key Message: **(A)** Wave has developed a transgenic mouse model (huADAR1 / huSERPINA1-Pi*Z) for recapitulating the liver AAT aggregation observed in homozygous PiZZ carriers. *In vivo* mouse experiments demonstrate that the platform can reach clinically relevant AAT serum concentrations (~ 11 µM for 7 days) after 3 loading doses with Q2W dosing resulting in > 11 µM serum A1AT. 80% elastase inhibition is achievable. **(B)** ~60% on-target editing is observed, suggesting that full transcript editing is not needed to address the lung manifestation of A1ATD. However, it is unknown if residual PiZ will contribute to liver manifestations associated with A1ATD; (C) Some off-target editing is observed, albeit at frequencies ~10-fold lower that on-target editing

(A)

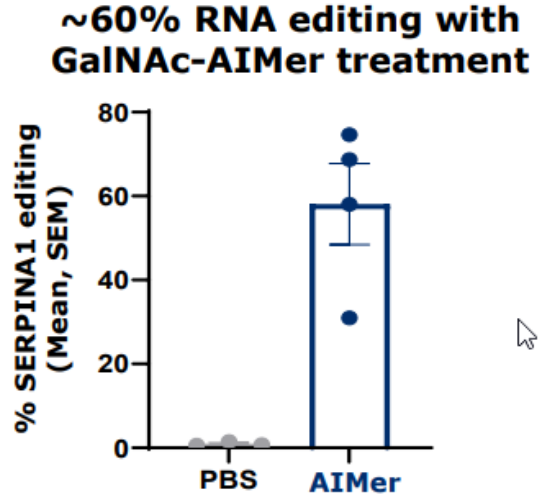


AIMer's Editing Efficiency 30-75% and Some Off-Target Editing

(B)

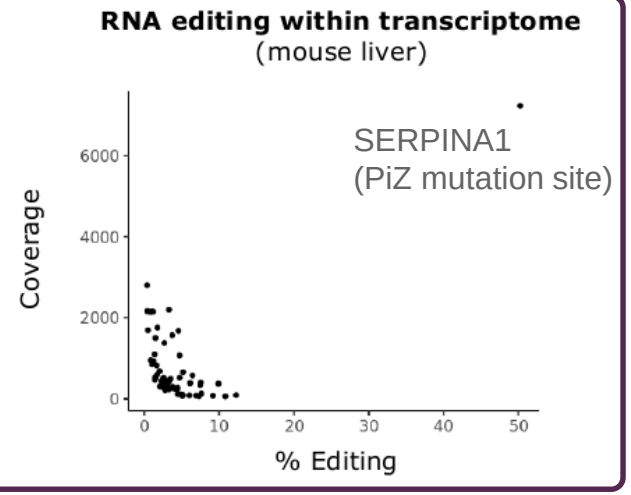
mRNA editing efficiency: Using a humanized mouse model 30% to 75% editing was observed in hepatocytes

Dosing: PBS or 10 mg/kg AIMer administered on days 0, 2, and 4 with sample collection on day 7



(C)

1. Some off-target editing was observed as indicated on the coverage vs. % editing plot albeit lower than PiZ transcript editing
2. No bystander editing was observed on the *SERPINA1* transcript



Sources: Wave Life Sciences Presentation September 28, 2021 - https://ir.wavelifesciences.com/static-files/4b998f29-712e-4077-8afd-37d86eb2a9ec m
Wave Life Sciences Presentation March 3, 2022 - https://ir.wavelifesciences.com/static-files/4e03e4de-0db7-45c6-8673-b855de4ca6b0

A1ATD Experience With VX-864 Calls into Question Translatability from Mouse to Human

The VX-864 Translational Data Temper Murine Data Enthusiasm

Key Message: VRTX's June 2021 discontinuation of its clinical-stage, small molecule misfolded protein corrector VX-864 for PiZZ A1AT may be an indicator for caution when considering translatability between A1ATD mouse models and humans with A1ATD. However: (1) a thorough PK/PD analysis for mouse and human would be needed to develop the most compete picture of comparability, and (2) an analysis of drug plasma exposure and plasma protein binding across the species would need to be included:

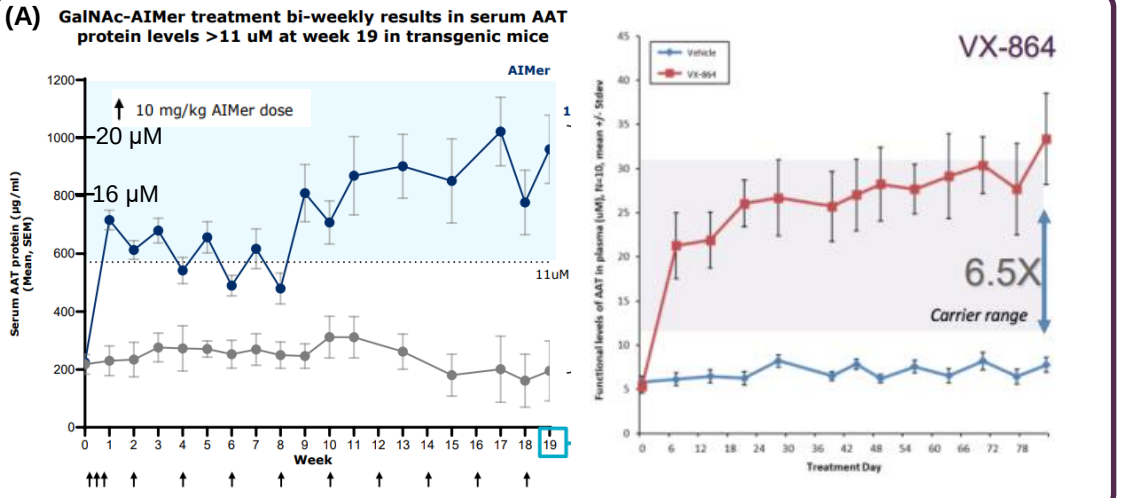
(A) WVE's AIMer increased serum AAT within the range 11 µM to 20 µM and this range is similar to values observed for VX-864. Additionally, baseline levels of AAT are comparable

(B) VX-864 clinical data over a 5X dose-range resulted in at most a 2.3-fold increase in functional AAT relative to baseline

Sources: WVE Corporate Presentation, March 3, 2022 (https://ir.wavelifesciences.com/static-files/4e03e4de-0db7-45c6-8673-b855de4ca6b0)
"CRISPR/Cas9-Mediated Targeted Gene Insertion of SERPINA1 to Treat Alpha-1 Antitrypsin Deficiency"
https://investors.vrtx.com/news-releases/news-release-details/vertex-announces-primary-endpoint-achieved-phase-2-study-vx-864

VRTX and WVE's Preclinical Data In Mouse Are Comparable

(A)



(B)

VX-864 Phase 2 Clinical Study, BID Dosing

Figure 3: Absolute mean functional and antigenic AAT levels at baseline and at day 28

	Functional AAT (micromolar)		Antigenic AAT (micromolar)	
	Baseline	Day 28	Baseline	Day 28
Placebo (N=7)	4.7	4.6	5.4	5.3
VX-864 100 mg q12h (N=10)	4.0	6.3	4.5	7.9
VX-864 300 mg q12h (N=9)	3.8	6.1	4.6	7.5
VX-864 500 mg q12h (N=18)	4.1	6.2	4.8	7.5

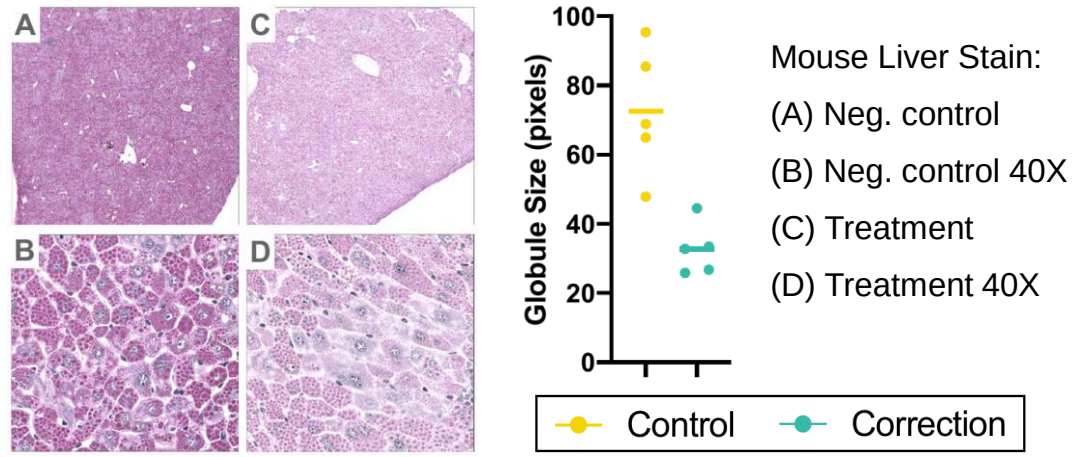
WVE vs. BEAM's Preclinical A1ATD Programs—Could AIMer in A1ATD Be a BE Hedge?

Preclinical Performance Comparable Between BEAM and WVE

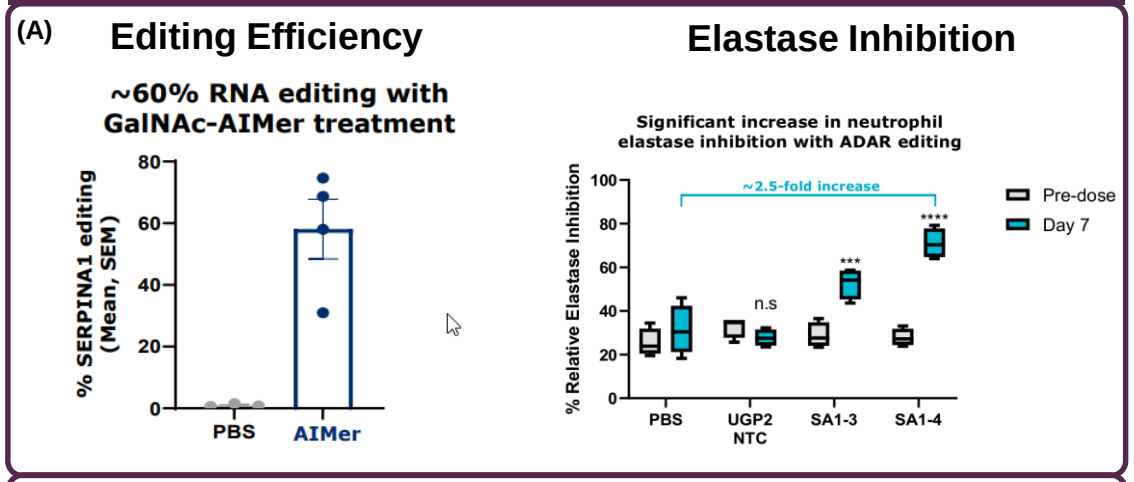
Key Message: Preclinical A1ATD genomic base editing data from Beam show lower editing efficiency and comparable elastase inhibition levels to Wave's AIMer program. However, Beam's therapy represent a potential one-and-done approach with the opportunity for selective pressure favoring edited cells. WVEs platform could be an alternative to editing the genome, which to date, has unknown safety implications

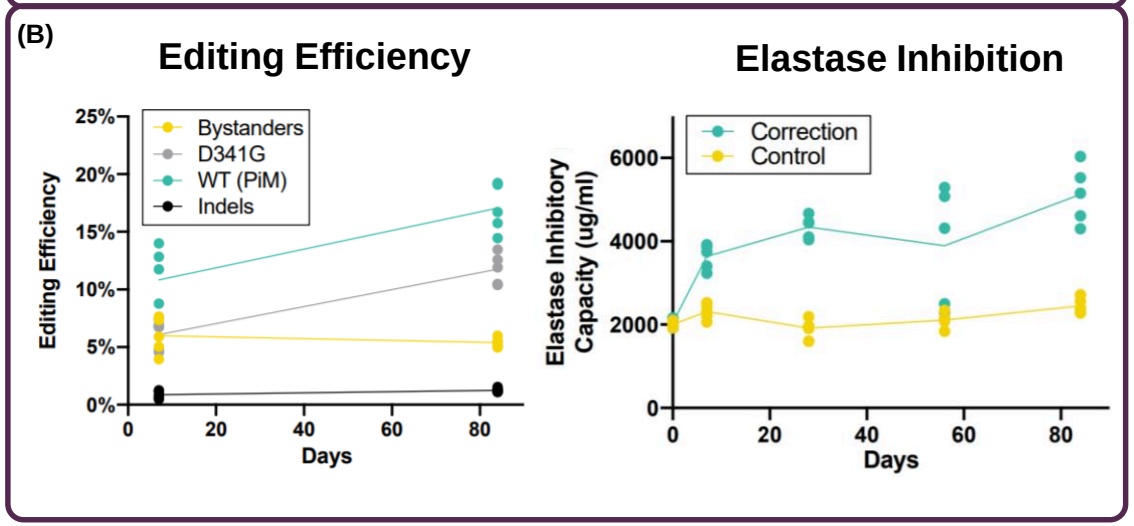
(A) Mice were dosed PBS or 10 mg/kg AIMer administered on days 0, 2, and 4 with sample collection on day 7

(B) A single dose of Beam's editor results in the formation of active PiM, D341G (bystander and productive edit), and 5% inactive bystander. Data's increase in editing efficiency with time suggest a potential survival advantage for edited hepatocytes relative to non-edited hepatocytes. This phenomenon may not be observed in the context of RNA editing if the background of PiZ in partially edited cells accumulates



Editing Efficiency and Elastase Inhibition for WVE and BEAM





Sources: https://beamtx.com/wp-content/uploads/2021/06/202005-ASGCT-2020-Alpha-1-Poster-vFinal.pdf, WVE Corporate Presentation, March 3, 2022 (https://ir.wavelifesciences.com/static-files/4e03e4de-0db7-45c6-8673-b855de4ca6b0)

See pages 73 - 74 for analyst certification and important disclosures.

AIMers Can Be Used to Disrupt Protein-Protein Interactions

mRNA editing approach to disrupt protein-protein interactions

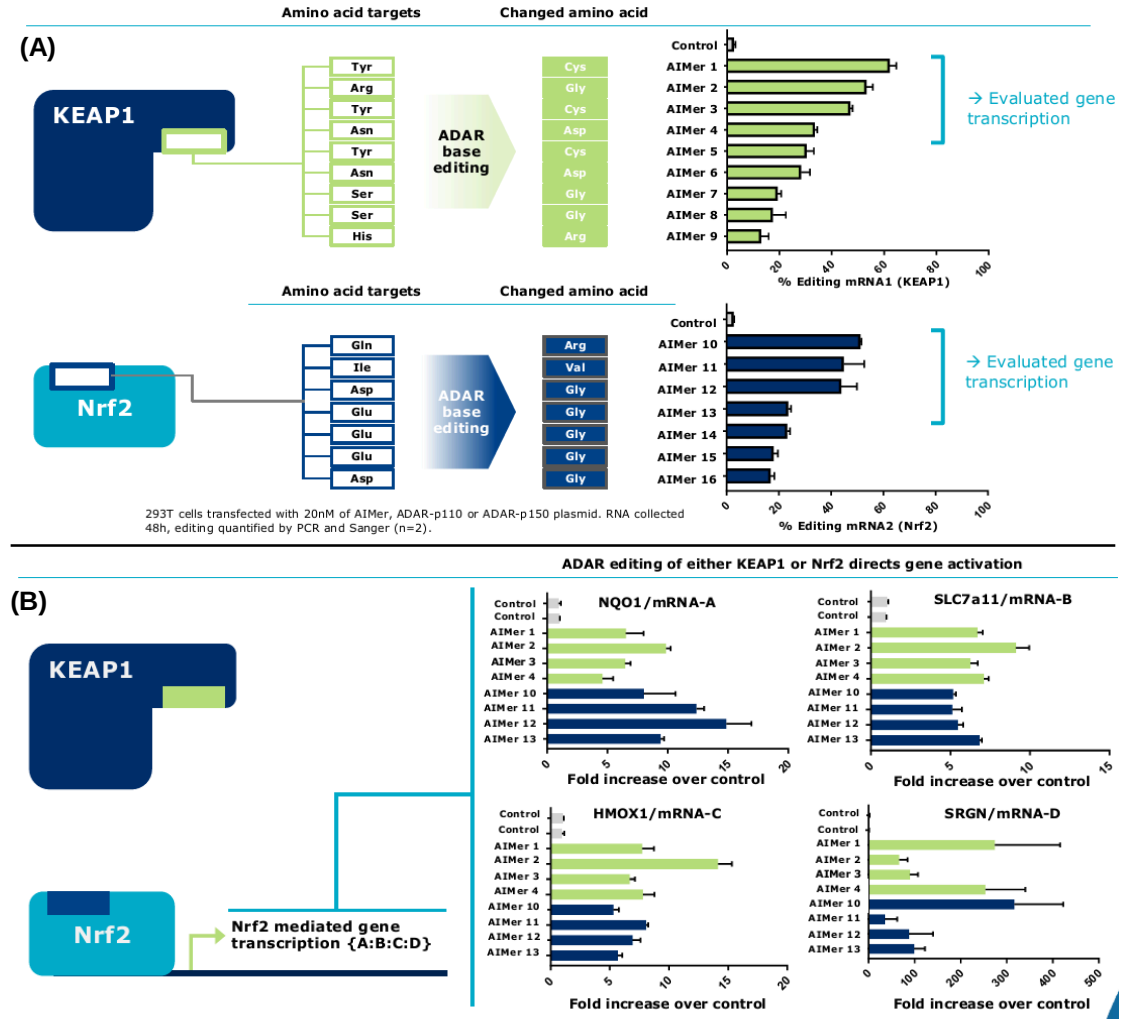
Key Message: Wave has established a series of mRNA edits that can be made in either KEAP1 or Nrf2 to disrupt protein-protein interactions. The downstream influence these mRNA edits have on Nrf2-regulated gene expression have been characterized for each AIMer generated. 5- to 200-fold increases in gene expression relative to control were observed:

(A) Wave has characterized a family of AIMer and their corresponding editing efficiency for targeting the mRNA of either KEAP1 or Nrf2

(B) Following the editing efficiency characterization as described in panel (A), the down-stream consequences of disrupting the KEAP1/Nrft2 interaction were measured using a Nrf2 gene transcription assay measuring the transcripts for NQ01, SLC7a11, HMOX1, and SRGN.

Source: WVE Corporate Presentation, March 3, 2022 (https://ir.wavelifesciences.com/static-files/4e03e4de-0db7-45c6-8673-b855de4ca6b0)

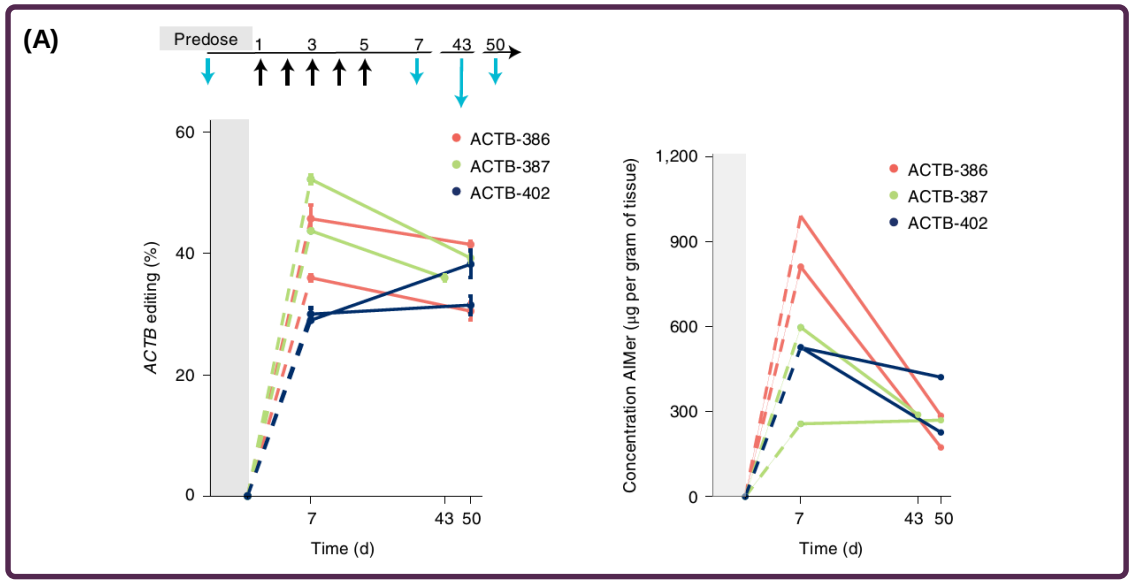
Editing efficiency / gene expression through RNA editing



Non-Human Primate Data Show Sustained (~50 days) RNA Editing in the Liver

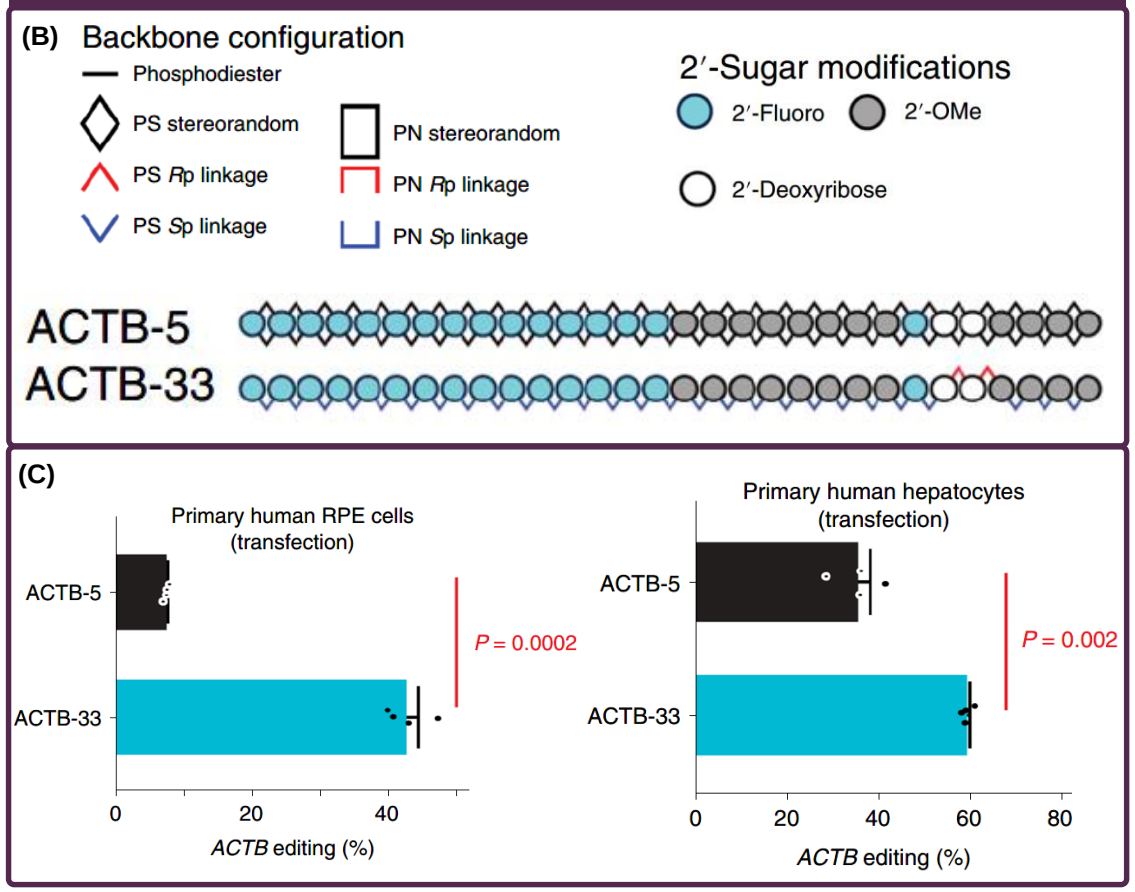
Proof of Mechanism Data In NHPs Look Encouraging

Key Message: (A) NHP data show sustained ACTB editing with a more rapid decline in liver AIMer concentrations over the same time period suggesting the dosing regimen used saturated the editing pathway; (B) WVE can access specific chemical modifications to tune the properties of the AIMers they construct; (C) the integration of stereo-specific control appears to confer greater editing efficiency relative to stereorandom PS



Source: https://pubmed.ncbi.nlm.nih.gov/35256816/

In Vitro Data Suggest Stereochemical Control Can Enhance Editing



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ProQR Therapeutics (PRQR) - Axiomer®

Executive Summary RNA Editing at ProQR—Looking Forward to the Data

Axiomer® platform appears primed for action—looking to the future for pre-clinical data disclosure:

1. The Axiomer platform utilizes synthetic oligos to bind with target RNA and recruit ADAR to install site-specific A>I mutations
2. The company has provided tabular data suggesting its platform can achieve 50% to 80% editing, but no off-target editing data have been provided
3. The Axiomer platform has been used to install A>I mutations in therapeutically relevant targets such as *hSERPINA1* and *hUSH2A (AAT and Usher syndrome, respectively)*

Eli Lilly (LLY) partnership to pursue 5 targets across the liver and nervous system:

1. The research collaboration focuses on the discovery, development, and commercialization of potential new medicines for genetic disorders in the liver and nervous system
2. Axiomer® RNA editing will be applied for up to five new drug targets with clinical development
3. ProQR will retain exclusive rights to R&D for genetic eye diseases.

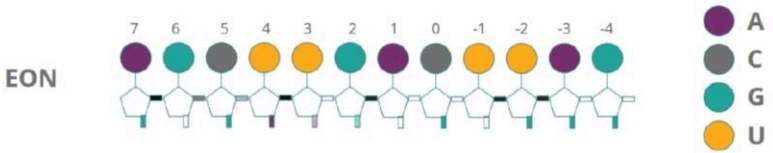
Axiomer® Platform Appears Primed for Action—Looking For Future Pre-Clinical Data

Axiomer Platform Can Access Contemporary Oligo Chemistry

Key Message: (A) ProQR is developing synthetic oligos (EONs) that can recruit endogenous ADARs for A>I (read as G) RNA editing; **(B)** The chemistry being leveraged to synthesize EONs access stereochemical control, ribose modifications, and sequence control; **(C)** Tabular data across several targets suggests the Axiomer platform can achieve 50% to 85%. However, no off-target editing data have been provided. Targets such as *hSERPINA1* and *hUSH2A* are relevant for diseases such as AAT and Usher syndrome respectively.

(B)

Patents broadly cover EON chemistry & chirality

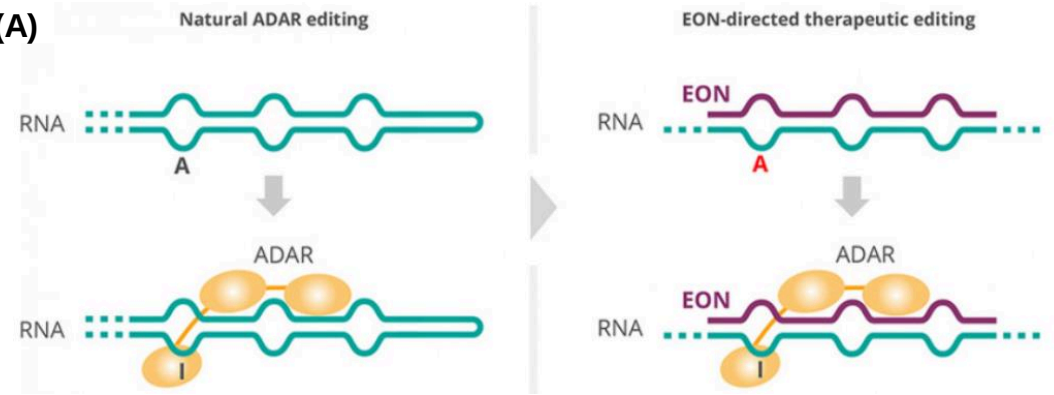


Aspect	Determined by	Modifications	Effects
Base	Target RNA	Mismatches and analogs	Improved PD
Ribose modification	ADAR structure	2'-H; 2'-OMe; 2'-MOE; 2'-F; 2'-NH2, LNA, TNA	Improved PK and PD
Linkage	ADAR structure	PO; PS; PN; MeP; UNA; PAc	Improved PK and PD

Source: https://www.proqr.com/files/2021-09/ProQR%20Axiomer%20Technology%20Overview.pdf

Editing Efficiency

(A)



(C)

Functional aim of editing	Target RNA	Editing up to*
Reverse G-to-A mutation	*GFP*	85 %
Reverse G-to-A mutation	*mIdua*	60 %
(None; WT target)	*mUsh2a*	80 %
Reverse G-to-A mutation	*hUSH2A*	50 %
Inactivate protease site	*hAPP*	50 %
Inactivate kinase site	*hEPHB3*	60 %
Inactivate kinase site	*hEPHA7*	60 %
Reverse G-to-A mutation	*hSERPINA1*	70 %

*ProQR data on file

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Private Companies Exploring RNA Editing

Private Companies Employing RNA Editing

Company	RNA Editing Platform	Strategy	RNA Editing Focus Areas	Source
ShapeTx	RNAfix	AAV-mediated delivery of a vector that (1) is transcribed to RNA; (2) the transcribed RNA pairs with a cellular RNA target; (3) the pairing recruits ADAR for A>I mutation	Tissues: Liver, CNS, and Eye Indications** mentioned: alpha-1 antitrypsin deficiency, parkinsonism, and Stargardt disease	https://www.shapetx.com/*
DTx Pharma	Fatty Acid Ligand Conjugated OligoNucleotide (FALCON™)	(1) The fatty acid conjugated oligo binds to the RNA target, and (2) recruit ADAR for A>I editing	Tissues: eye, neuromuscular, and muscle Indications: pan/genetic retinitis pigmentosa	https://dtxpharma.com/
Korro Bio	Oligonucleotide Promoted Editing of RNA (OPERA™)	(1) Synthetic oligo pairs with the target RNA and (2) pairing activates ADAR recruitment for A>I editing	Tissues: liver, CNA, and eye Indications: lead candidate for alpha-1 antitrypsin deficiency	https://www.korrobio.com/
ADARx	Not disclosed	NA	Tissues Liver, Heart and CNS Indications: Alpha-1 Antitrypsin Deficiency	https://www.adarx.com/
LOCANAbio	CORRECTX™	Mentions ADAR-based RNA editing main focus appears to be on RNA knockdown and splicing	neurodegenerative, neuromuscular and retinal diseases	https://locanabio.com/ https://locanabio.com/wp-content/uploads/2021/06/RNA-society-2021.pdf
EdiGene	LEAPER™ (Leveraging endogenous ADAR for programmable editing of RNA) and LEAPER™ 2.0	AAV-mediated delivery of a vector that (1) is transcribed to RNA; (2) the transcribed RNA pairs with a cellular RNA target; (3) the pairing recruits ADAR for A>I mutation	Beta-hemoglobinopathies, Mucopolysaccharidosis Type 1	https://www.edigene.com/in_vitro_therapies/ https://pubmed.ncbi.nlm.nih.gov/31308540/ https://pubmed.ncbi.nlm.nih.gov/35145313/

*Data were previously disclosed on the ShapeTx website but are no longer available

**These indications were discussed previously on the ShapeTx but were not necessarily indicated as part of the therapeutic pipeline

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Shape Therapeutics [PRIVATE]

Executive Summary Shape Therapeutics

ShapeTx focuses on employing optimized gRNA, encoded in AAV vectors, to support delivery, expression, and endogenous ADAR-catalyzed RNA editing:

1. ShapeTx has developed high throughput screening methodology to optimize gRNA vectors for ADAR-based RNA editing
2. Ideal editing kinetics have been identified to enhance the selection process
3. Insights have been gained to avoid bystander editing

Indications that have been explored in preclinical models include: AAT, parkinsonism, and Stargardt disease

1. **Familial Parkinson's Disease**: RNAfix identified high levels of on-target A>I conversion with some ~5% off-target bystander editing
2. **Stargardt Disease**: ShapeTx's screening methodology identified gRNA constructs that effectively mitigate the influence of G nucleotide dampening of target edit. Resulting optimal gRNA shows 75% RNA editing with ~2% bystander effect
3. **Alpha-1 Antitrypsin**: The identified gRNA for promoting the SERPINA1 E342K mutation. This resulting gRNA results in ~10% bystander editing. The E342K mutation, also known as the Z dysfunctional allele has a prevalence of about 70,000 in the US (ZZ homozygous)

AAVid^{TM} is enabling ShapeTx to expand tissue tropism:

1. 10^{11} variant screens are being conducted to identify new AAVs with specific tissue tropism
2. Follow-on evaluation in NHPs is supporting the development of vectors that target the CNS

Shape's platform is now being implemented in a partnership with Roche for undisclosed targets in Alzheimer, Parkinson, and the rare disease areas

Shape Employs AAV and Encoded gRNA for ADAR-Based RNA Editing

High-Throughput Screening to ID RNA Editing Sequences

Key Message: Shape has develop screening methods to identify candidate non chemically-modified gRNA that can (1) hybridize with target RNA; (2) recruit ADAR for A>I editing. The screening methodology is sold as an approach to optimize gRNAs that maximized on-target editing and minimize off-target editing.

Challenges in RNA editing:

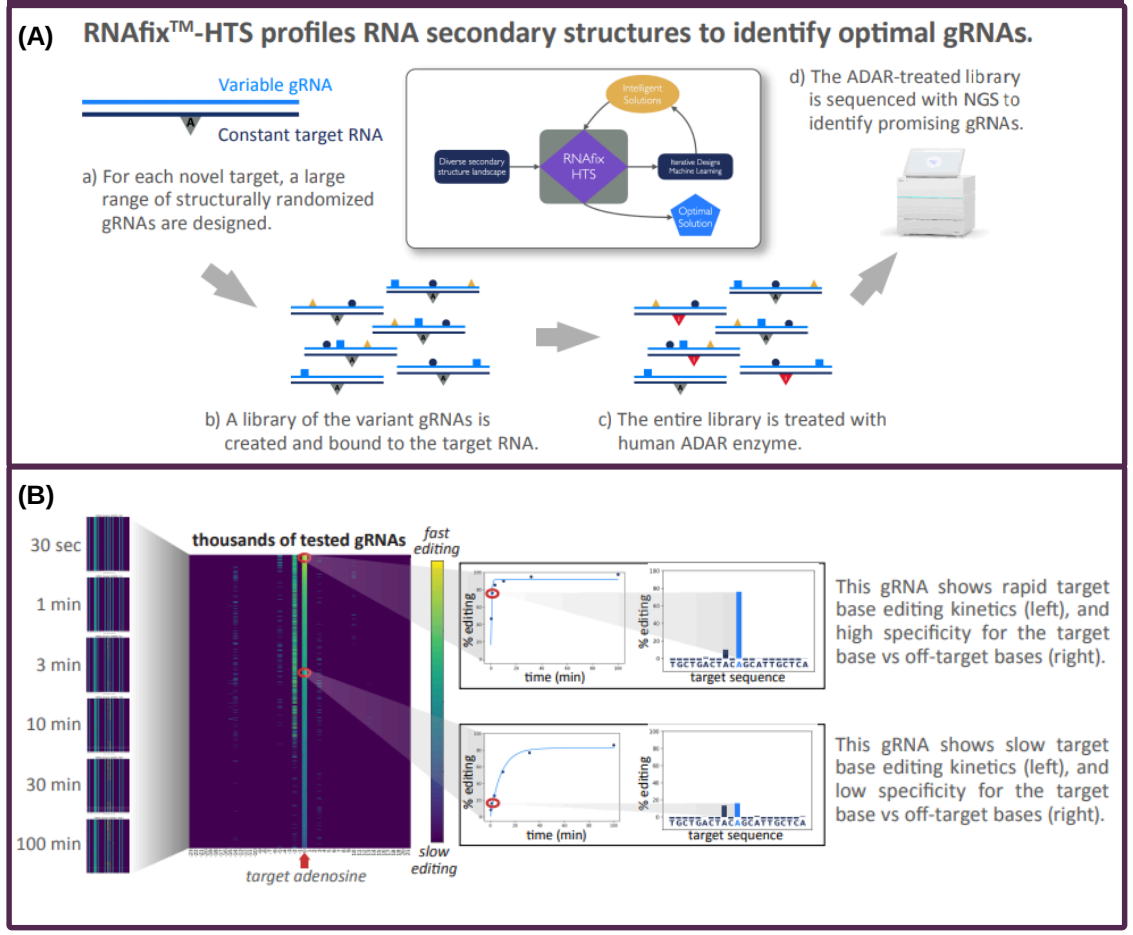
- Gene-encoded gRNAs must be ~60 nucleotides long to recruit endogenous ADAR. However, the longer the gRNA length, the greater the possibility of off-target A>I editing
- Placement of a single A-C mismatch between the gRNA and target adenosine can be used to increase efficiency and specificity, but this comes at a cost of flexibility for choosing a clinically-meaningful target

RNAfix to address challenges:

- (A) High-throughput screening is conducted to identify lead sequences that, in the presence of ADAR, catalyze the target edit
- (B) Sequence hits identified under (A) are screened for the appropriate kinetics to support high on-target editing and low off-target editing

Source: ASGCT 2021 Poster, Deep Screening of Guide RNAs Enables Therapeutic RNA Editing with Endogenous ADAR

Screening Workflow and RNA Editing Kinetics



Characterization of ADAR1/2 Activity may Be Needed for Evaluating Off-Target Editing

Multiple ADARs May Contribute to Observed RNA Editing

Key Message: (1) RNA editing mediated by gRNA and ADAR may have contributions from both ADAR1 and ADAR2; (2) these two enzymes show expression in the same tissues with ADAR1 expression exceeding those of ADAR2; (3) ADAR isoform distribution has an unknow effect on ADAR-based RNA editing

ADAR molecular biology:

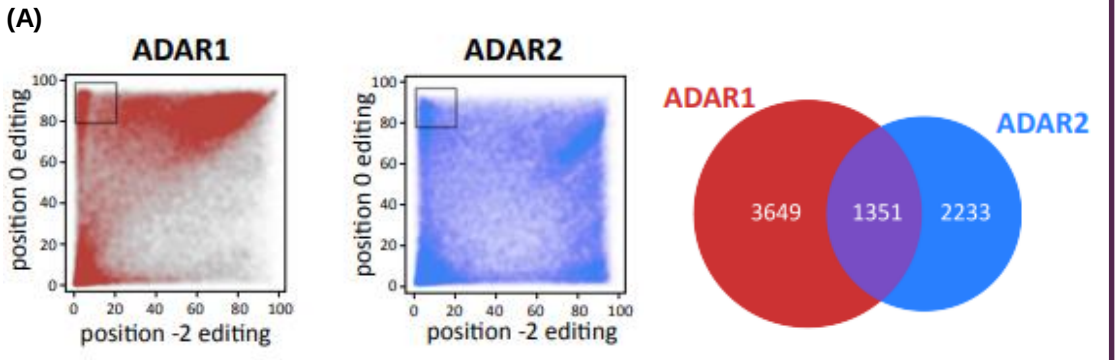
ADAR1 and ADAR2 are widely expressed in human tissues whereas ADAR3 is brain-specific (unknown catalytic activity)

(A) A given gRNA construct may show activity for RNA editing mediated by both ADAR1 and ADAR2. The data displayed exemplify the importance of characterizing the activity of both ADAR1 and ADAR2 as gRNA-mediated on- and off-target editing may be orthogonal for the two enzymes. Shape has the ability to map out the on- and off-target RNA editing for both ADAR1 and ADAR2

(B) ADAR2 is expressed in similar tissues to ADAR1; albeit at lower frequencies. Interestingly, the data presented show that several ADAR isoforms are expressed. The influence of the distribution of isoforms on editing has not been described

Sources: ASGCT 2021 Poster, Deep Screening of Guide RNAs Enables Therapeutic RNA Editing with Endogenous ADAR, https://www.nature.com/articles/srep14941

Analysis of RNA Editing ADAR1/2 and Tissue Distribution



Evaluation of on- and off-target editing for the Parkinson's-disease-relevant transcript LRRK2 G2019S

Rundown of Therapeutic Targets Being Developed: Neurology, Ocular, and Metabolic/Liver

Indications Are Diverse and Therapeutic Constructs Are Improving

Key Message: Indications being explored include familial Parkinson's disease, Stargardt disease and alpha-1 antitrypsin deficiency. Some of the RNA edits performed to prosecute the target overcome previously identified challenges in ADAR-based RNA editing, including an edit-adjacent G nucleotide motif known to lower editing efficiency (see Stargardt example)

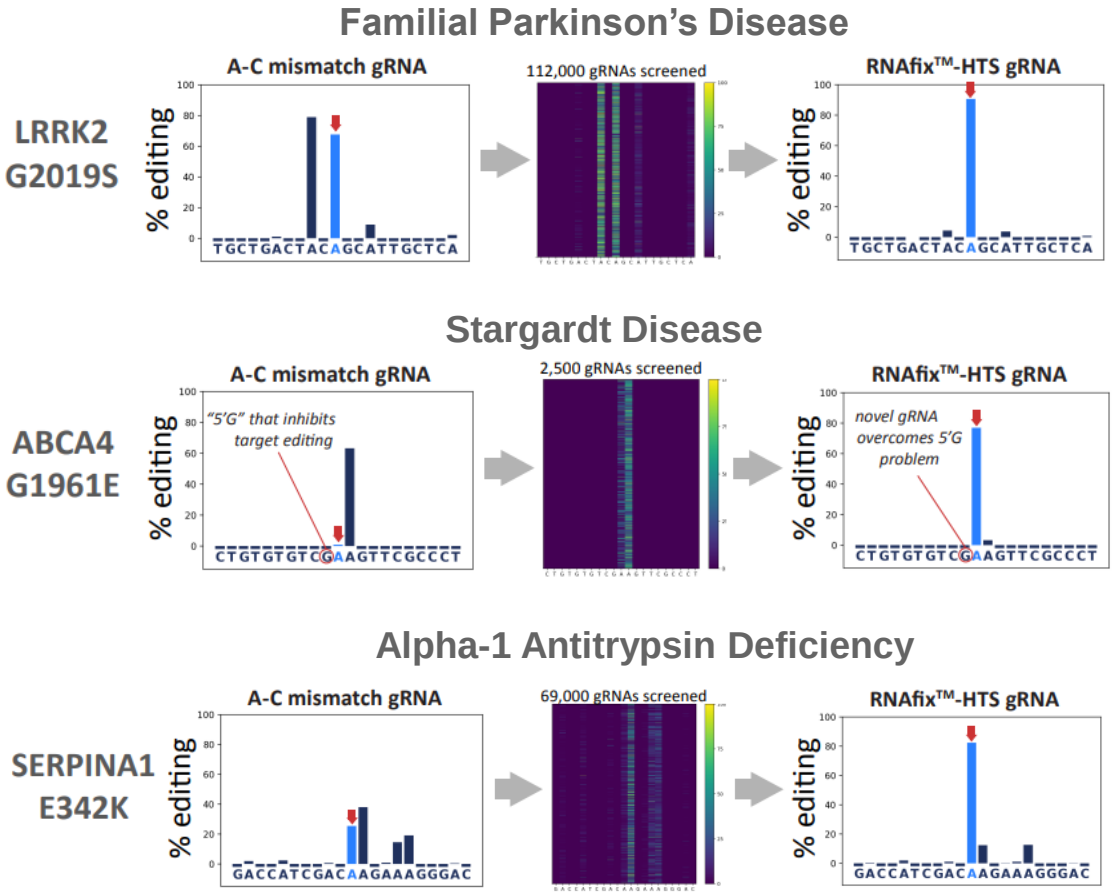
Familial Parkinson's Disease: RNAfix identified high levels of on-target A>I conversion with some ~5% off-target bystander editing

Stargardt Disease: Shape's screening methodology identified gRNA constructs that effectively mitigate the influence of G nucleotide dampening of target edit. Resulting optimal gRNA shows 75% RNA editing with ~2% bystander effect

Alpha-1 Antitrypsin: The identified gRNA for promoting the SERPINA1 E342K mutation. This resulting gRNA results in ~10% bystander editing. The E342K mutation, also known as the Z dysfunctional allele has a prevalence of about 70,000 in the US (ZZ homozygous)

Sources: : ASGCT 2021 Poster, Deep Screening of Guide RNAs Enables Therapeutic RNA Editing with Endogenous ADAR, https://doi.org/10.1016/S0140-6736(05)66781-5

Indications and Corresponding Constructs' Editing Efficiencies



Circular adRNAs Show Enhanced RNA Editing – Further Optimization *In Vivo* Is Needed

CadRNA Approach Holds Promise but Need Improvement *In Vivo*

Key Message: Shape has reported that circular ADAR recruiting guide RNAs (cadRNAs) can be used to confer greater levels of mRNA editing, putatively through abrogation of adRNA-disruptive exonuclease activity. CadRNA-based *in vivo* editing of the mPCSK9 transcript does not achieve the depth of editing observed for VERV's base editor in similar model systems.

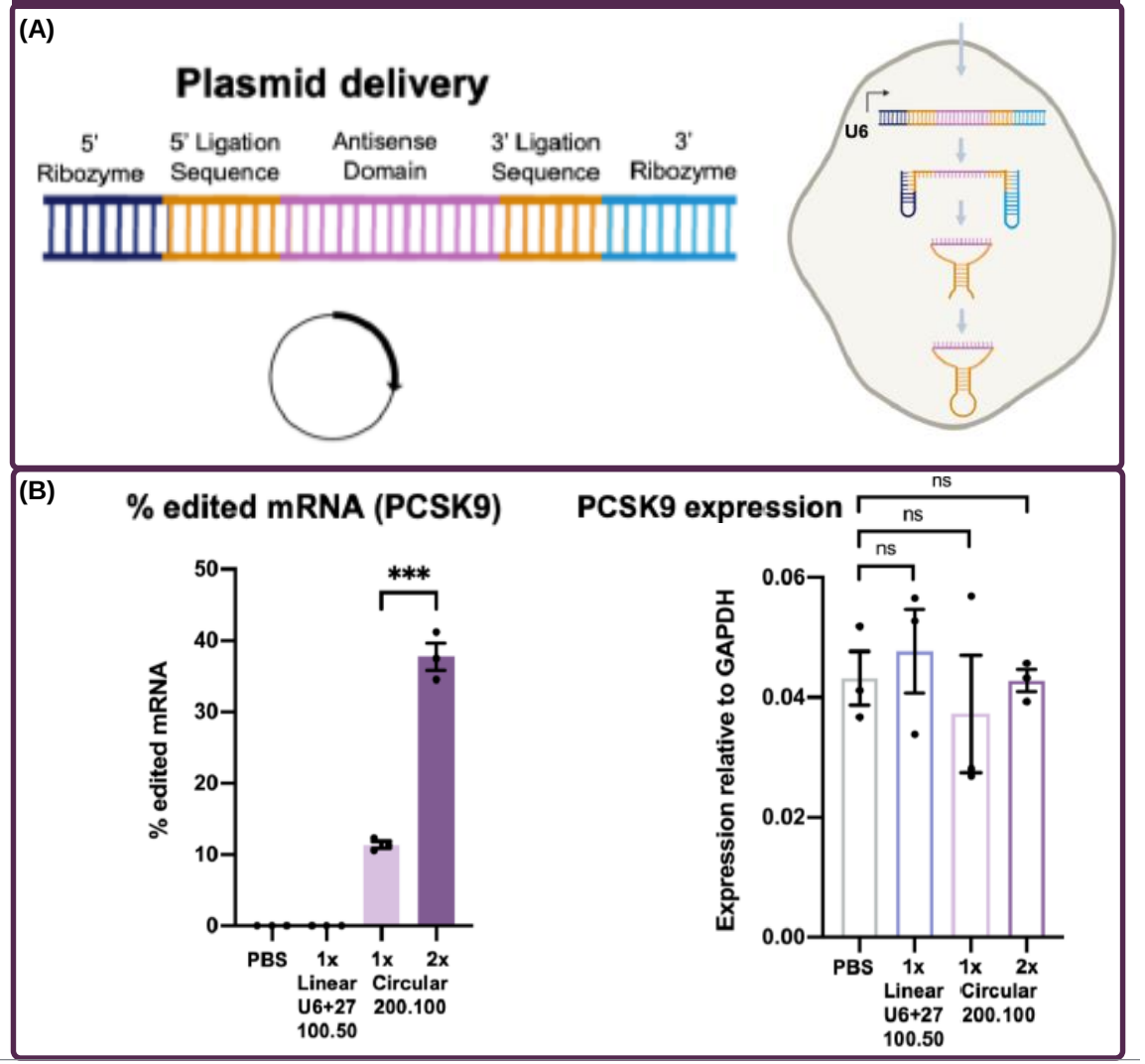
Shape's cadRNA platform:

(A) Circular RNA, resistant to cellular exonucleases, is produced *via* the delivery of a circular plasmid encoding the ADAR-active sequence flanked by the twister ribozyme sequences. Post transfection and expression (1) the ribozyme sequences undergoes autocatalytic cleavage; (2) RtcB ligates the two ends of the pre-cadRNA; (3) mature cadRNA pairs with the target mRNA and recruits ADAR for RNA editing

(B) 38% RNA editing in mouse PCSK9 transcript in (m = C57BL/6J mice) livers with no observable change in PCSK9 expression. Framing the PCSK9 results: VERV reported 70% liver base editing in mouse. Additionally, IONS' antisense oligonucleotide ION449 (LICA technology) demonstrated >90% reduction in LDL-cholesterol in humans after subcutaneous administration (baseline LDL-C, 100 and 190 mg/dL)

Sources: https://doi.org/10.1101/2021.01.12.426286, https://www.nature.com/articles/s41586-021-03534-y.epdf?sharing_token=HsonYNLZxtKY14BoswRIRtRgN0jAjWel9jnR3ZoTv0M6Hd6FaG8LKZpFk4zRG3bRk_k-yu6JL-SBSnxGMXRwXfIxZsV5lzyijNLy4qu_cSv4Njqsxu4TZYFLKqINGhvzwDQdUC2kvLRcc5A7mJcY0h8UigbTDJ4CDJRIu00lw70%3D, https://ir.ionispharma.com/news-releases/news-release-details/promising-new-data-ionis-antisense-medicine-targeting-pcsk9

Plasmid Processing and *In Vivo* Editing



The AAVid™ Platform Screens for Tissue Tropism—Experimental Confirmation Pending

Next Step - Demonstrate Tropism in Human-Relevant Systems

Key Message: Shape is pushing into the AAV5 engineering space by leveraging large (~10^{11} variants) screens of capsid variants. Data demonstrate mapping of tropism drivers and candidate prioritization but lack experimental evidences of targeted tissue specificity

Major challenges in engineering AAVs with high tissue tropism: (1) low diversity in capsid libraries; (2) selection of modalities in a translationally-relevant framework

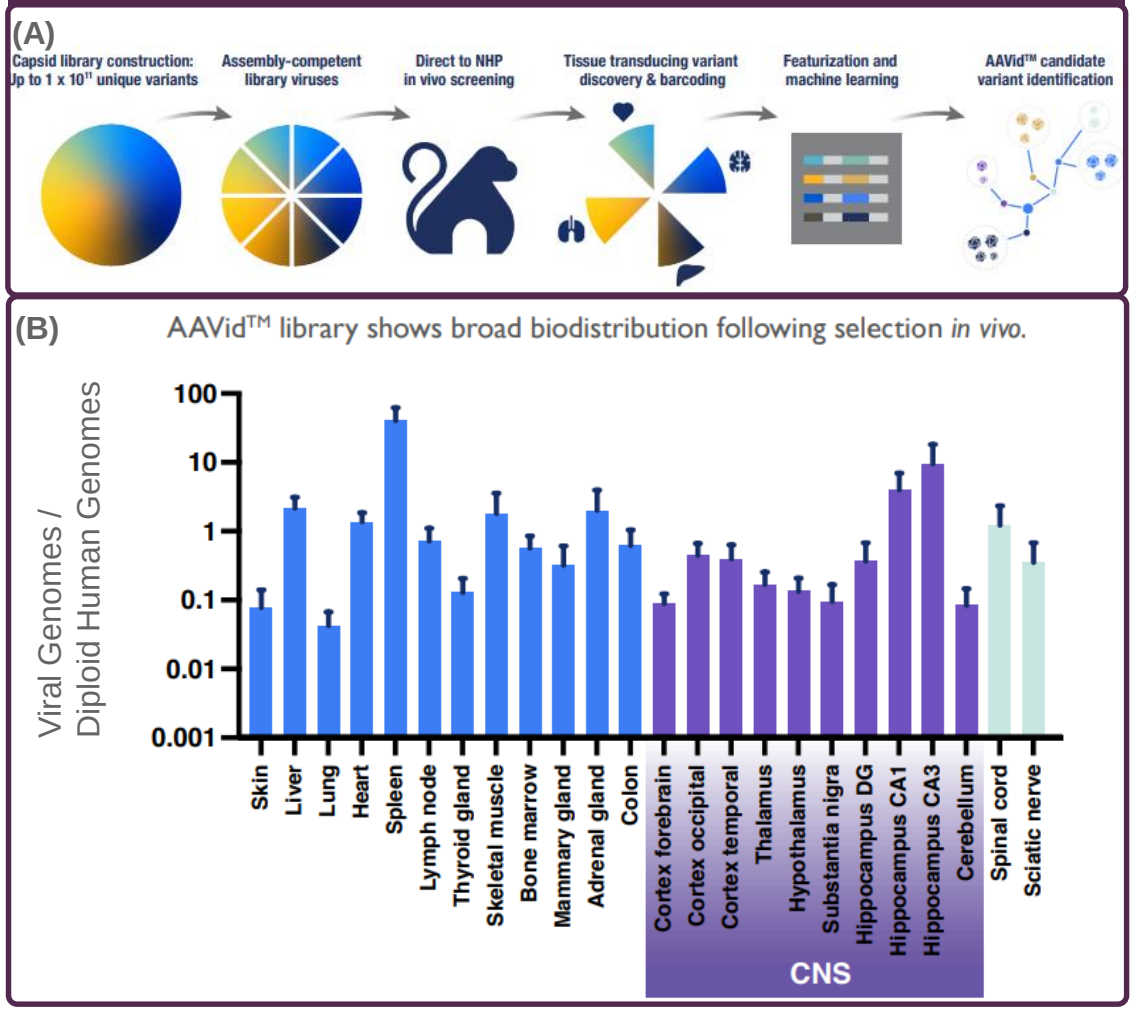
Shape's approach to enhance AAV engineering: Screening diverse combinatorial variant libraries in non-human primates

- Focus on 9-mer sialic acid-binding amino acid sequence part of the capsid region in the vector AAV5. The previously identified 9-mer binding sequence mediates tropism and can influence immunogenicity by creating escape mutants
- Starting pool contains 100 billion (9-mer has a theoretical 512 billion permutations) amino acid variants

(A) Workflow **(B)** Viral genome levels found in necropsied NHPs (N=2)

Sources: ASGCT 2021, AAVidTM: A Platform for Intelligently Engineering AAV Capsids from Massively Diverse Libraries, https://pubmed.ncbi.nlm.nih.gov/25410855/

Work Flow and *In Vivo* Transfection



Roche (RHHBY) Partnership to Focus on RNA Editing in CNS and Rare Disease Indications

Shape is drawing attention from premier big pharma players

- Shape will use RNAfix™, and potentially the AAVid™ delivery platform for next-generation tissue-specific adeno-associated virus-mediated disposition, for the development of RNA therapies for targets in the Alzheimer, Parkinson, and rare disease areas
- Shape will focus on preclinical research and leverage its AI-driven methodology to deliver development candidates to Roche in support of worldwide commercialization
- Specific targets have not been disclosed

Source: https://www.globenewswire.com/news-release/2021/08/24/2285515/0/en/Shape-Therapeutics-enters-into-a-strategic-research-collaboration-with-Roche-to-advance-breakthrough-AAV-based-RNA-editing-technology-for-neuroscience-and-rare-disease-indications.html

GUGGENHEIM

EdiGene [PRIVATE]

Executive Summary EdiGene

EdiGene Is Using Circular adRNA (cadRNA) to Recruit Endogenous ADAR for A-to-I Editing in the Transcriptome:

1. From the laboratory of Prof. Wensheng Wei of Peking University, LEAPER and LEAPER 2.0 (cadRNA) was developed to support editing in the transcriptome
2. A recent Nature Communications publication outlines the foundational technology parameters needed to optimize LEAPER 2.0, including: (1) mediation of bystander editing by developing a cadRNA that results in A bulges; (2) flanking AC repeats to enhance editing; and (3) examples highlighting the utility of cadRNA over linear constructs

First-Generation LEAPER™ Showed Glycosaminoglycan (GAG) Lowering in Hurler Syndrome Mouse:

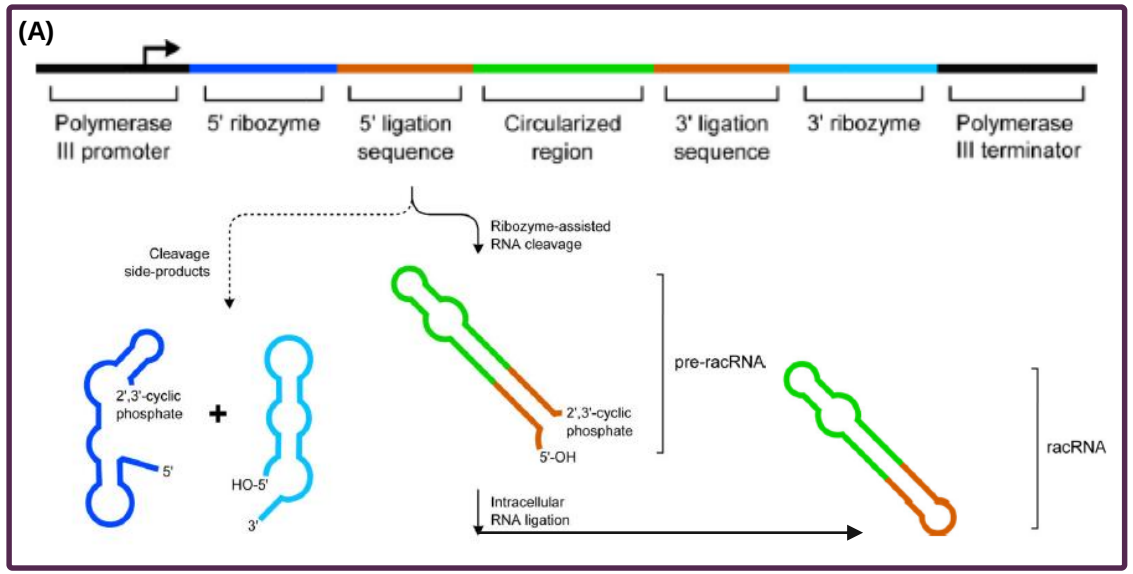
1. EdiGene is pushing into the mucopolysaccharidosis type I (aka Hurler syndrome) therapeutic area by developing LEAPER constructs for restoring functional alpha-L-iduronidase
2. Initial mouse data look encouraging. However, GAG lowering may be needed in multiple organ compartments, including the CNS, to fully address the breadth of tissues impacted by the condition

Circular arRNA LEAPER™ 2.0 Approach Accessible by EdiGene

LEAPER 2.0 From EdiGene Founder Prof. Wensheng Wei

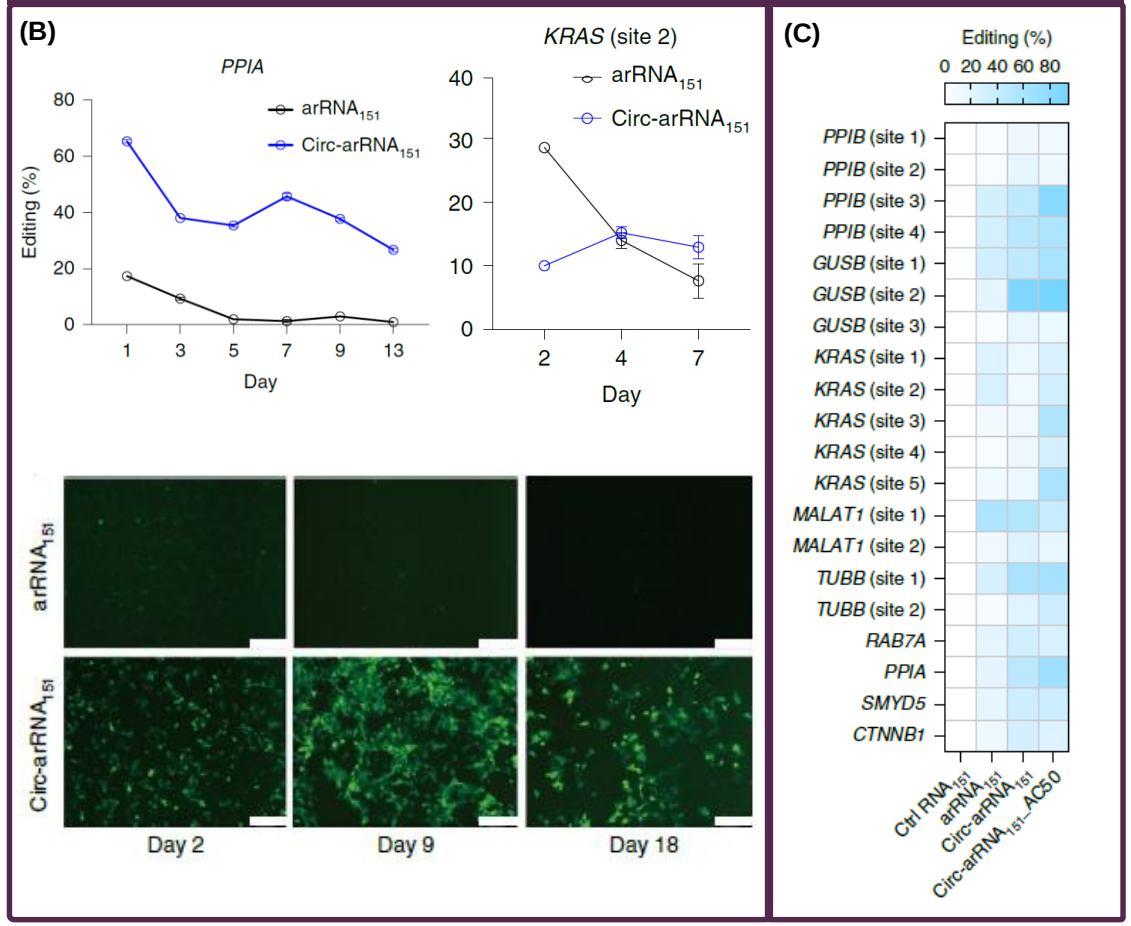
Key Message: Circular ADAR-recruiting RNAs (arRNAs) enhance RNA A-to-I(G) editing by stabilizing the construct relative to linear arRNA

(A) Sequences needed to form Circ-arRNA in cellular systems; (B) Editing efficiency and durability is enhanced for Circ-arRNA$_{151}$ relative to arRNA151 for the *PPIA* transcript and to a lesser extent for the *KRAS* transcript; (C) Circ-arRNA with flanking AC repeats (AC50) access greater levels of editing for select sites in HEK293T cells



Source: https://pubmed.ncbi.nlm.nih.gov/30962542/, https://pubmed.ncbi.nlm.nih.gov/35145313/, https://www.edigene.com/media/89.html

Circ-arRNA Greater and Longer Editing Relative to Linear arRNA



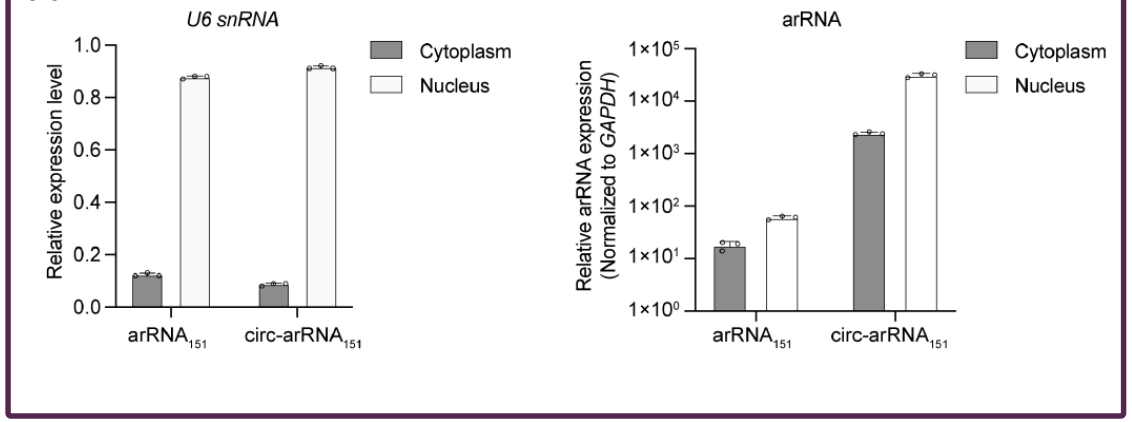
Circ-arRNA$_{151}$ Localizes to the Cytoplasm and Nucleus – AAV Delivery Achievable *In Vitro*

Circ-arRNA Partitions to Both Cytoplasm and Nucleus

Key Message: Circ-arRNA promotes transcriptome editing in both the cytoplasm and the nucleus (in mammals ADARs are primarily localized to the nucleus https://pubmed.ncbi.nlm.nih.gov/23273215/) and initial experiments demonstrate deliverability of the platform via AAV.

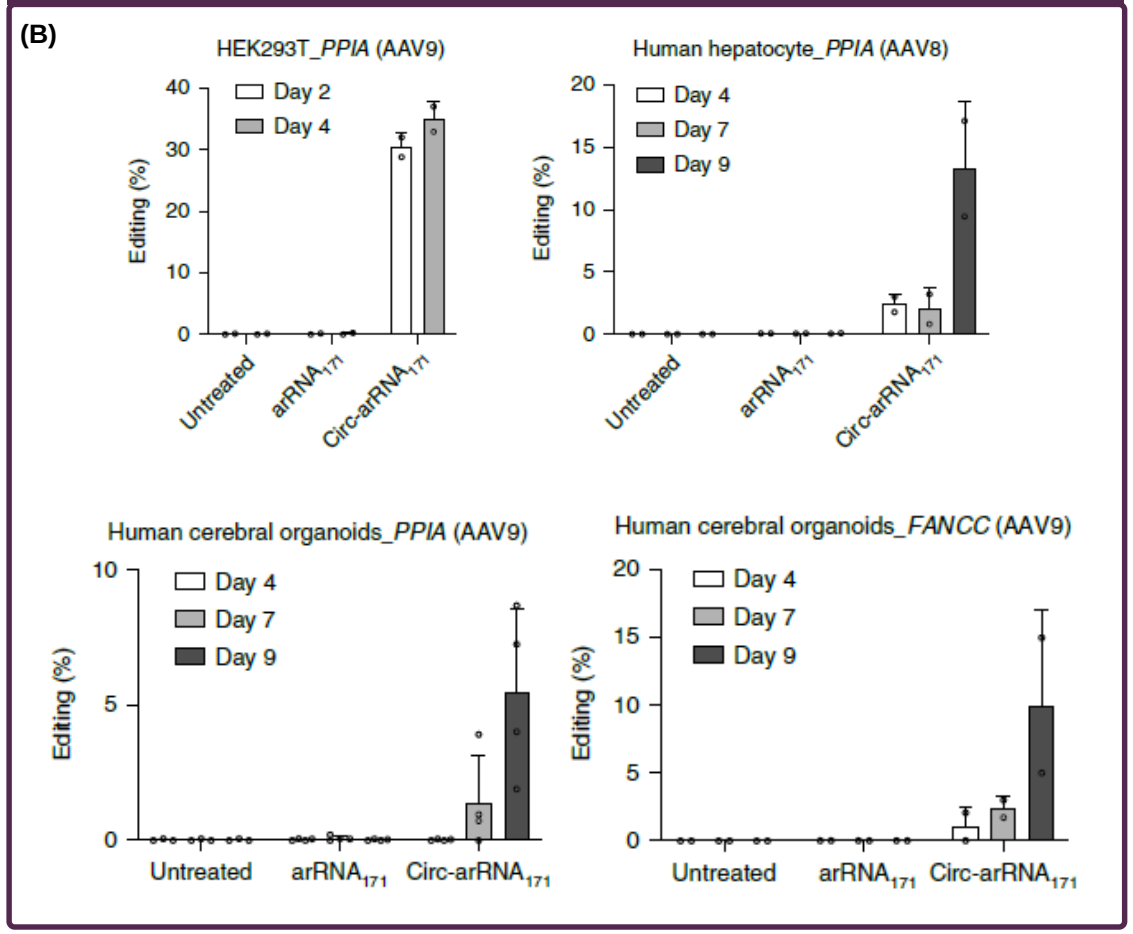
(A) Relative to linear arRNA$_{151}$, circ-arRNA$_{151}$ shows a similar distribution between the cytoplasm and nucleus with some bias towards nucleus partitioning

(B) Data suggest AAV-mediated delivery of linear arRNA cannot support RNA editing whereas Circ-arRNA shows an increase in editing with time



Source: https://pubmed.ncbi.nlm.nih.gov/35145313/

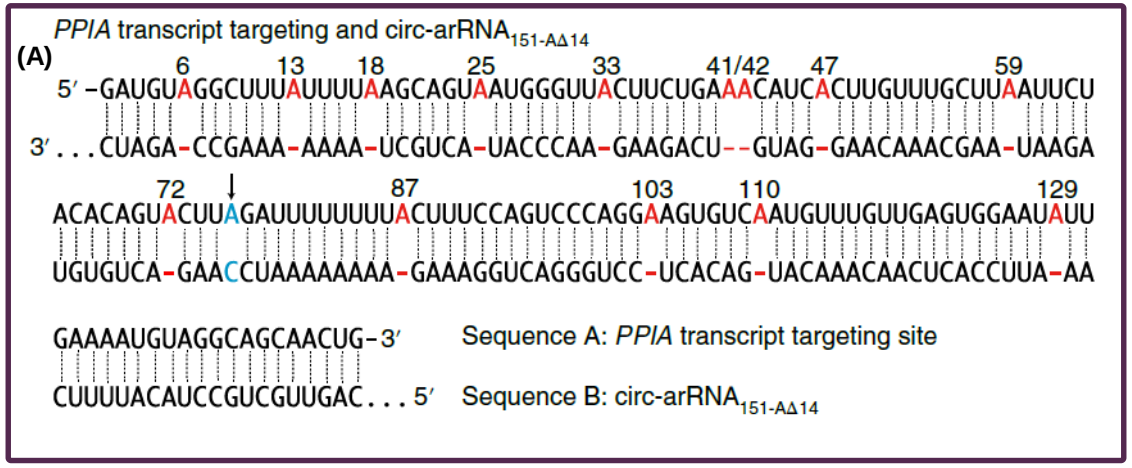
AAV-Mediated Deliver of Circ-arRNA Achievable *In Vitro*



Circ-arRNA$_{151}$ Off-Target < First-Generation ADAR2$_{DD}$ but Shows Some Bystander Editing

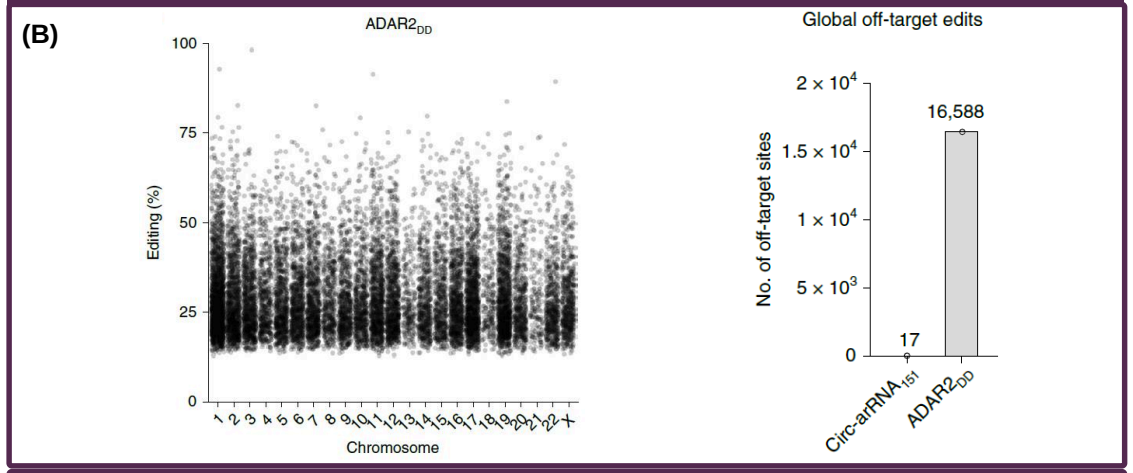
Incorporation of A Bulges Mediates Bystander Editing

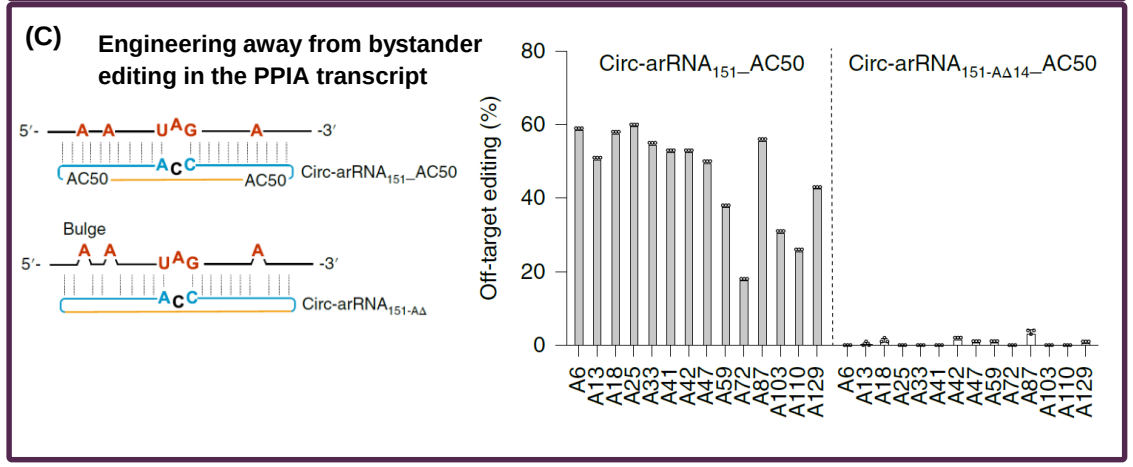
Key Message: (A) Target strand (5'-to-3') contains adenines "A" that are susceptible to bystander editing (red) and the targeting (3'-to-5') Circ-arRNA can include specific uridine "U" deletions "-" to induce A bulges; (B) off-target editing in the transcriptome is greatly reduced by using the Circ-arRNA approach relative to the addition of ectopic ADAR2$_{DD}$ with an appropriate adRNA; (C) Circ-arRNA_AC50 that does not contain U deletions to form A bulges at bystander editing sites results in higher off-target editing.



Source: https://pubmed.ncbi.nlm.nih.gov/35145313/

17 Potential Off-Target Edits Observed in the Transcriptome





EdiGene's First-Generation LEAPER™ Showed GAG Lowering in Hurler Syndrome Mouse

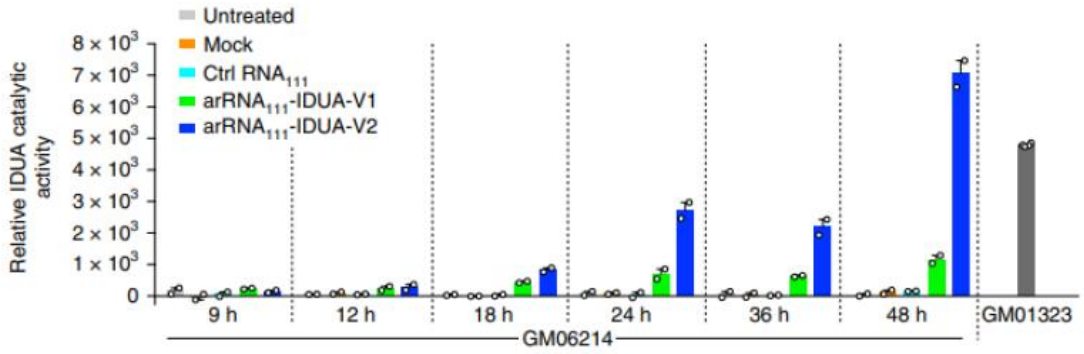
Teed Up – But Addressing Multiorgan Symptomatology Pending

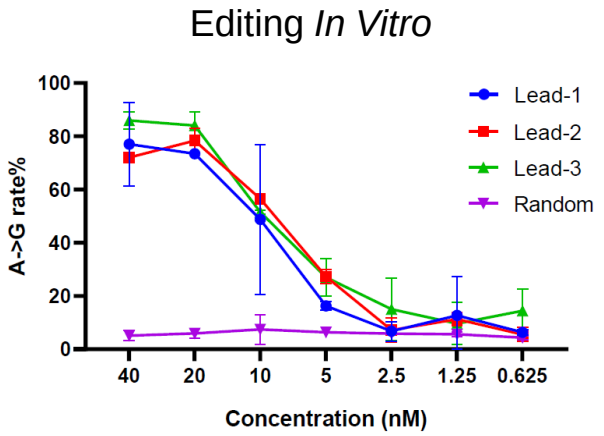
Key Message: (1) LEAPER is a short oligo < 100 nt that (2) can be delivered *via* plasma or synthetic oligo system; (3) bind to target RNA; and (4) recruit endogenous ADAR to support A-to-I(G) editing. Initial example in Hurler Syndrome demonstrates interest in lysosomal storage diseases but begs the question how this approach will address the multitude of organs affected by the condition including the CNS.

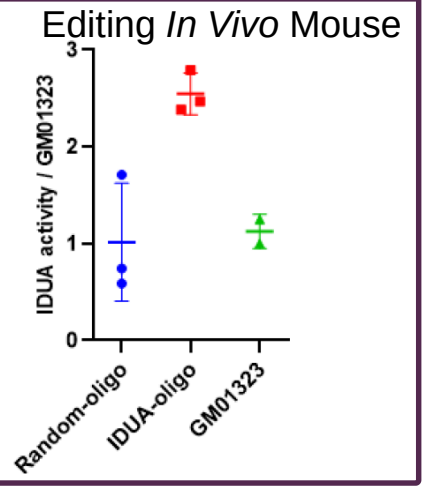
(A) Primary Hurler patient cells (GM06214) treated with arRNA111-IDUA-V2 showed increase activity of IDUA the enzyme (mutated IDUA when unedited lacks the ability to metabolize glycosaminoglycans (GAGs) leading to the accumulation of GAGs throughout the body)

(B) Editing efficiency can reach 80% at 40 nM concentrations and mouse data show increase in IDUA activity

W402X (c1205 G>A) Mouse Model Shows Restoration of IDUA







Source: ASGCT Presentation 2020 A Novel Oligonucleotide-based RNA Base Editing Approach for the Treatment of Hurler Syndrome, May 12th, 2020

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