UNLOCKING THE POTENTIAL OF INNOVATIVE EDITING OLIGONUCLEOTIDES (EONS)

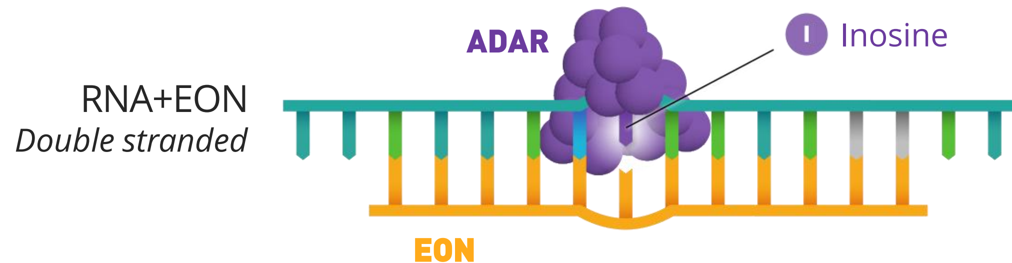
to Address Liver Originated Disorders

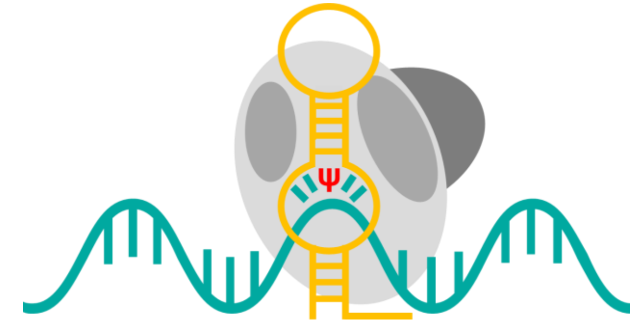
Gerard Platenburg, CSO and co-founder at ProQR

TIDES USA - May 9, 2023

RNA toolbox – editing platform technologies

Axiomer® and Trident® in development by ProQR





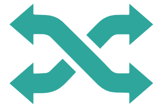
Axiomer® A-to-I editing

- Exploiting endogenous ADAR
- Recruited by synthetic Editing Oligonucleotide (EON)
- I is translated as a G, allowing to target G-to-A mutations
- Specific, potent, and stable by design
- Thousands of G-to-A mutations described in literature

Trident® U-to-Ψ editing

- Exploiting endogenous pseudouridylation machinery
- psEON adopts a hairpin structure with a guiding sequence ultimately recruiting the machinery
- Specifically target PTC mutations (~11% of all known disease-causing mutations)
- Broad applicability to various diseases caused by PTCs

Axiomer® RNA-editing platform technology



Versatile

- Ability to target multiple organs and a wide range of diseases with numerous applications
- Potential to include protective variants
- Designed to target a variety of RNA species (mRNA, miRNA, lncRNA)

Safety

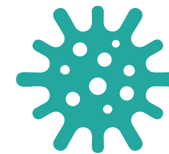
- No permanent changes
- No irreversible DNA damages and less risk of permanent side effects

High specificity

- Highly targeted therapeutic with potential to minimize off-target effects and reduce the risk of adverse reactions

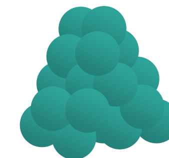
Transient

- Provide a long-lasting therapeutic effect that does not require frequent dosing
- Potential to target diseases for which permanent changes would be deleterious



No viral vector

- No risk of immunogenicity or capacity limitation due to the vector
- Efficient development and faster production increase the chance to reach market



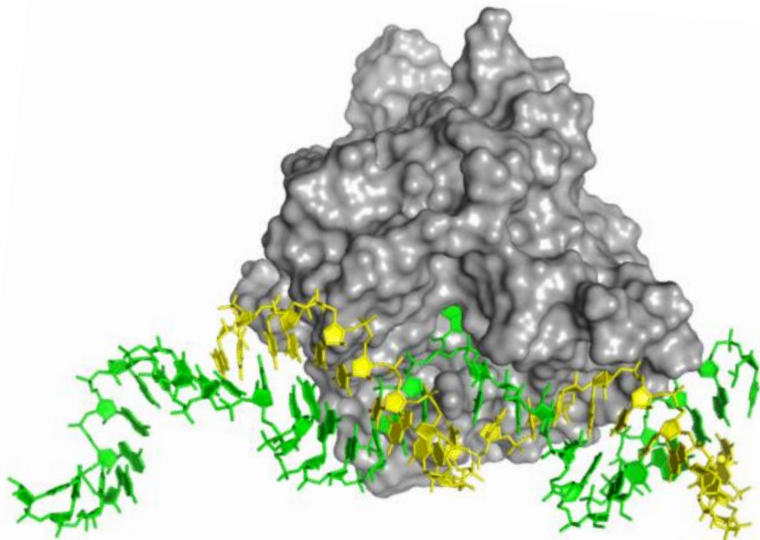
Endogenous ADARs

- Leverage body's potential to treat disease
- Less risk of off-target effect vs. exogenous ADARs

ADAR: Adenosine deaminase acting on RNA, mRNA: messenger RNA, miRNA: microRNA, lcRNA: long non-coding RNA

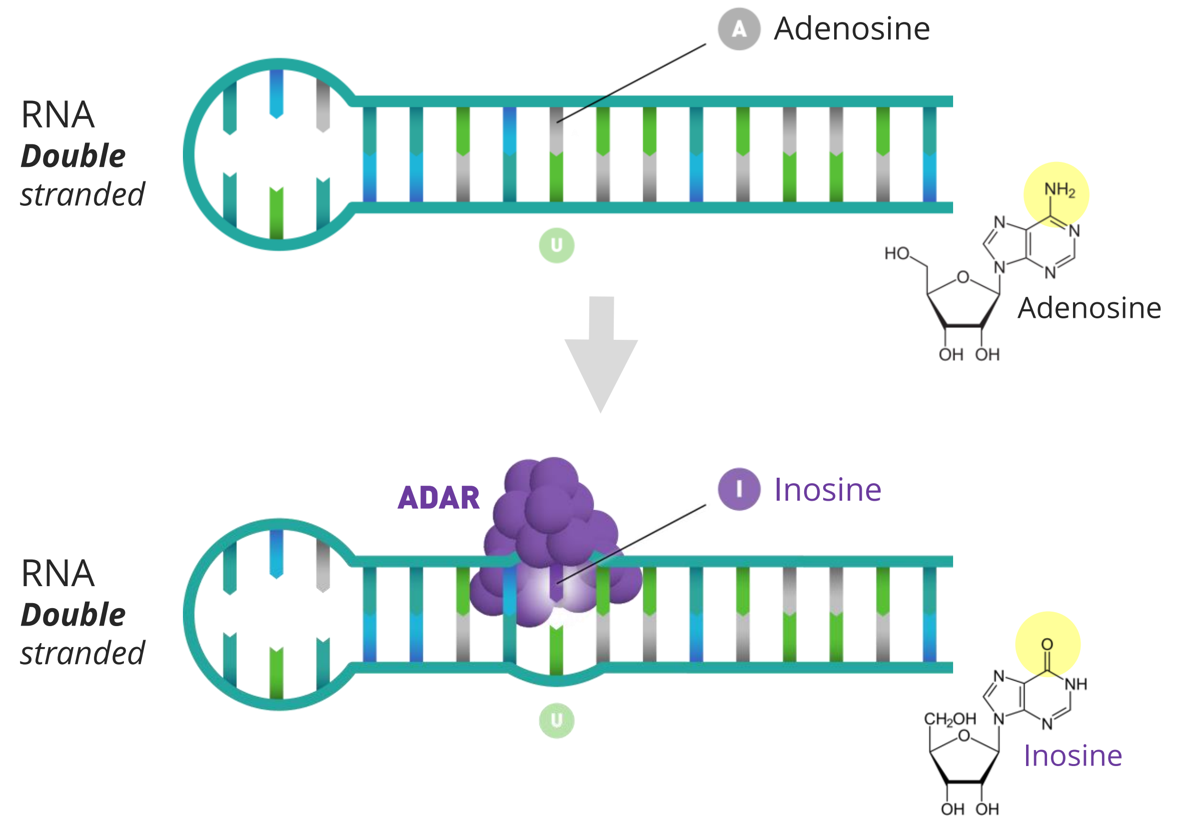
What is ADAR editing?

ADAR *(Adenosine Deaminase Acting on RNA)*



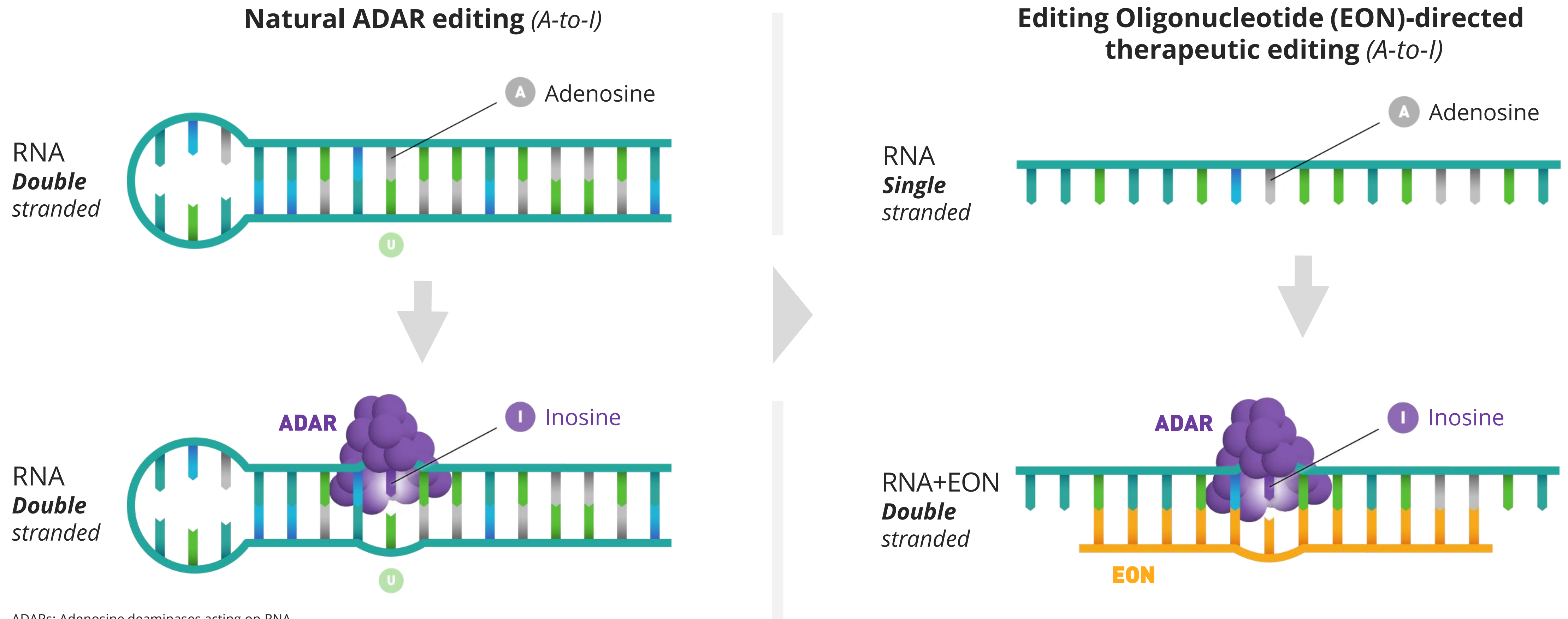
Enzyme that performs specific form of natural RNA editing, called **A-to-I editing.** During A-to-I editing an **A nucleotide (adenosine)** is changed into an **I nucleotide (inosine)**

Natural ADAR editing *(A-to-I)*



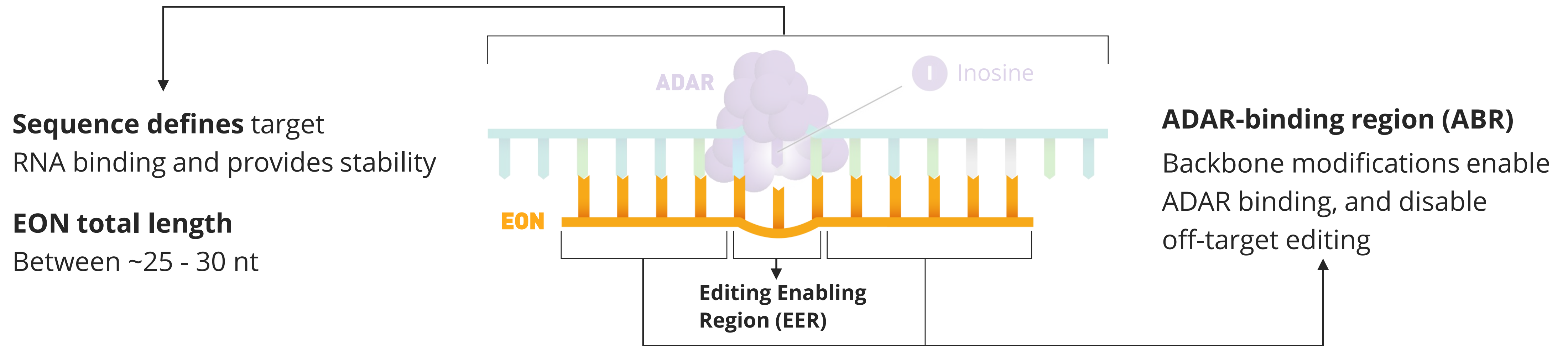
Axiomer® EONs unlock cellular machinery potential to treat diseases

By attracting ADARs and allowing highly specific editing

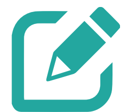


ADARs: Adenosine deaminases acting on RNA.

Driving the development of optimized EONs for therapeutic use

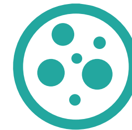


Optimized sequence and chemistry define functionality


Increase editing efficacy

Bring metabolic stability

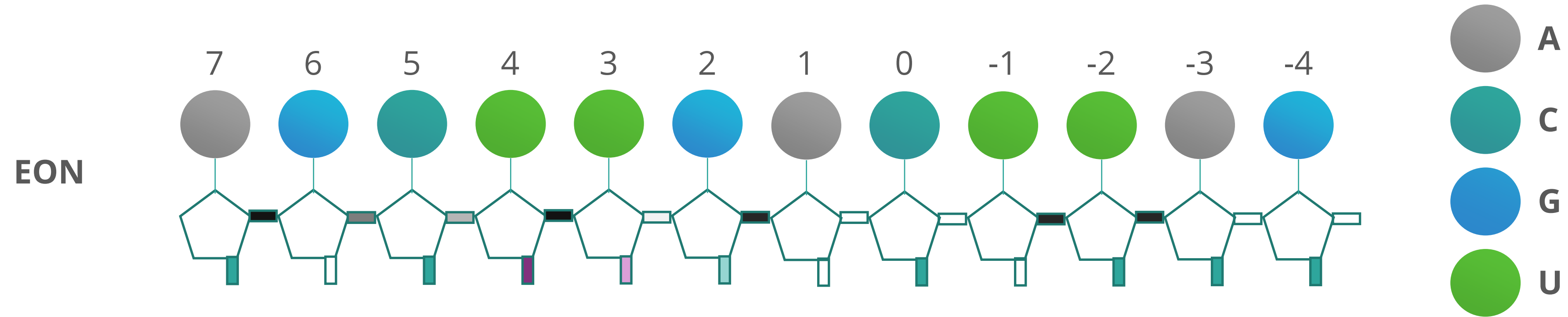
Prevent off-target ('bystander') editing


Ensure bioavailability (cell and tissue uptake)

Offer safety and tolerability at therapeutic doses

ADAR: Adenosine deaminase acting on RNA, EON: Editing oligonucleotide, Nt: nucleotides

Accelerating program advancement with focus on design principles



Aspect	Determined by	Modifications	Effects
Base	Target RNA	Mismatches and analogs (**dZ**)	Improved PD
Ribose modification	ADAR structure	2'-H, 2'-*O*-Me, 2'-MOE, 2'-F, 2'-NH_2, LNA, TNA, UNA, 2',2'-diF, FANA	Improved PK and PD
Linkage	ADAR structure	PO; PS; **PN**; **PMe**; PAc	Improved PK and PD

ADAR: Adenosine deaminase acting on RNA, EON: Editing oligonucleotide, PD: pharmacodynamic, PK: pharmacokinetic

ProQR leading research to optimize EONs for therapeutic use

Modification of the orphan base

in the EER confirm superiority of dZ base

Positive impact of neutral linkage modifications in the ABR

Improvement in editing potential with PN and PMe linkages on EONs

Structure–activity relationship (SAR) assessment

interrogating the impact of single change to define guiding principles

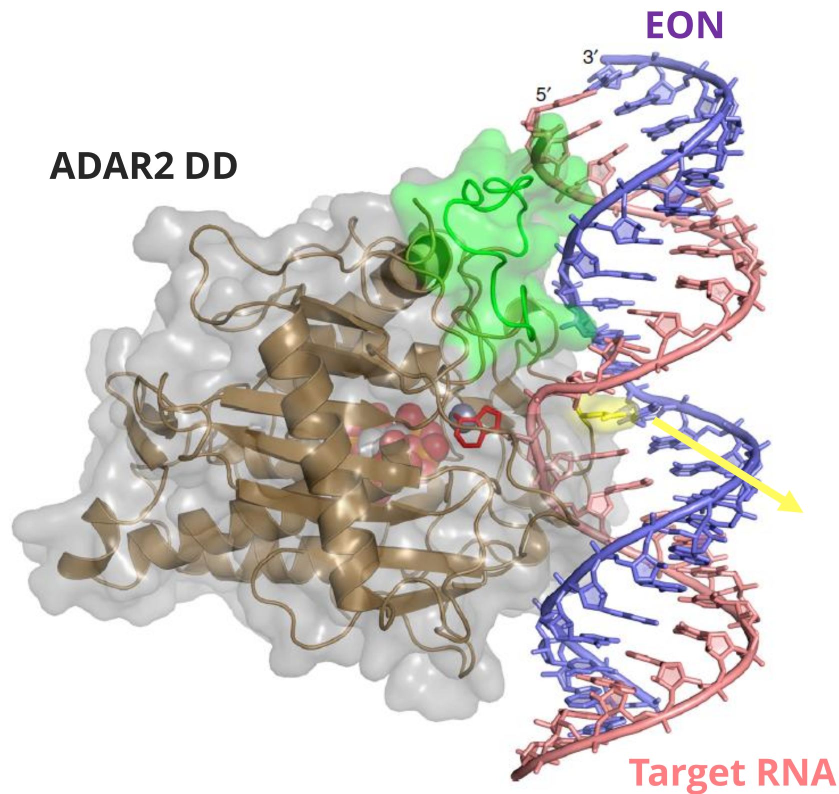
Modification in the Editing Enabling Region (EER)

Cytidine analogs as orphan base

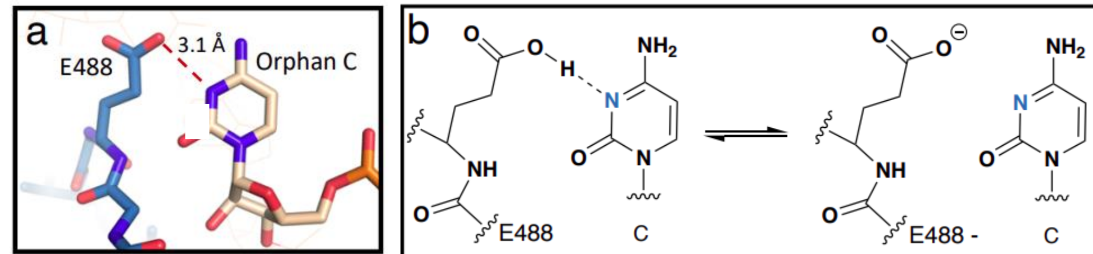
A single base modification of the EER increases ADAR activity

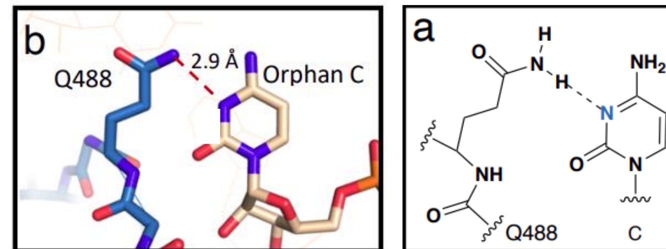
PROQR – UC DAVIS COLLABORATION

dZ base mimics E488Q mutation in ADAR2 causing hyperactivity

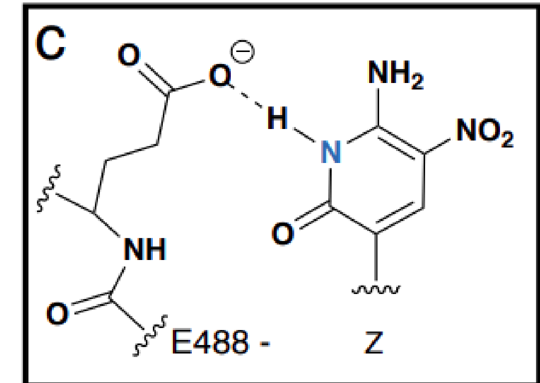


Protonation dependent hydrogen bond – pH dependency





Protonation independent hydrogen bond



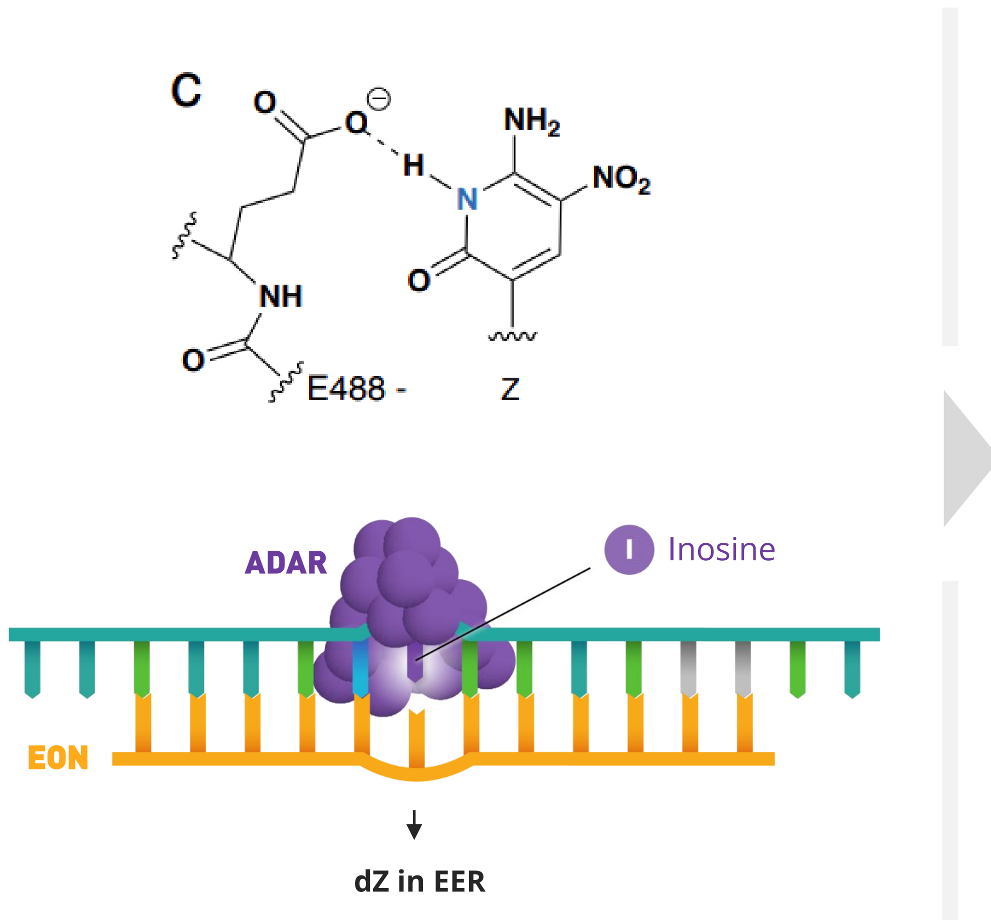
dZ base (dZ)

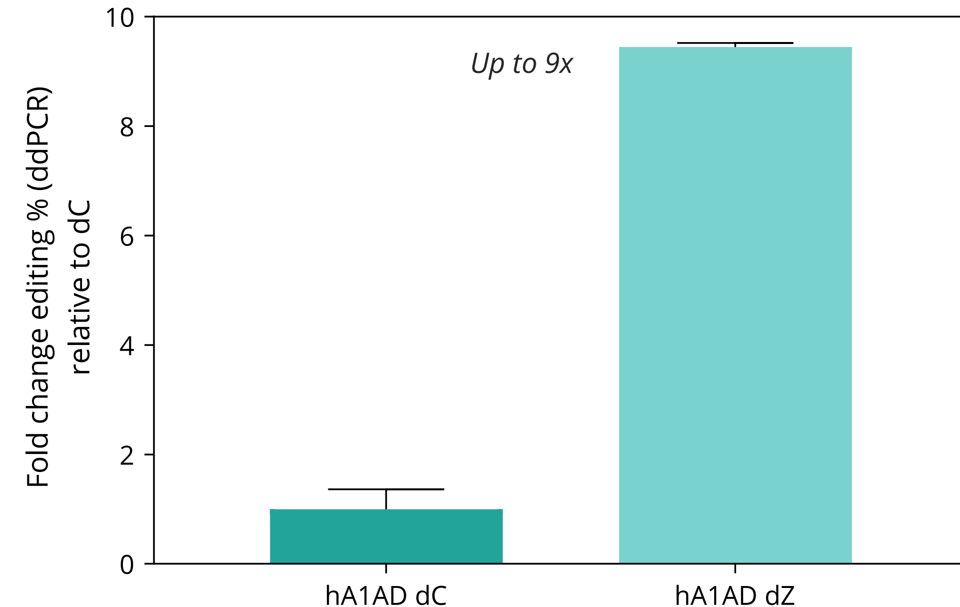
Metthews 2016, Nature Structural & Molecular Biology

Doherty et al., 2021, JACS, ProQR – UC Davis collaboration

dZ in the EER improves editing of *SERPINA1* E366K in A1AD patient hepatocytes

PROQR – UC DAVIS COLLABORATION





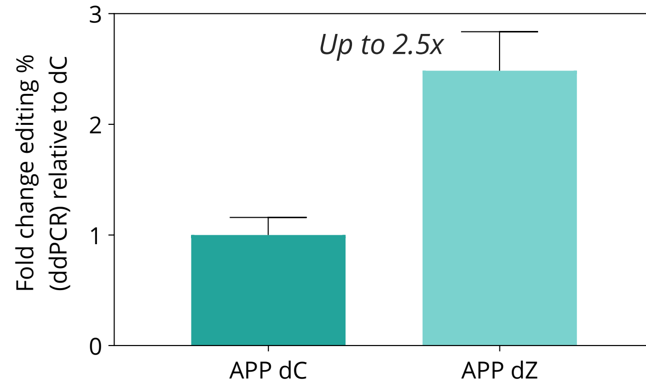
Improved editing obtained for several systems

dZ improves editing in different cell types

PROQR – UC DAVIS COLLABORATION

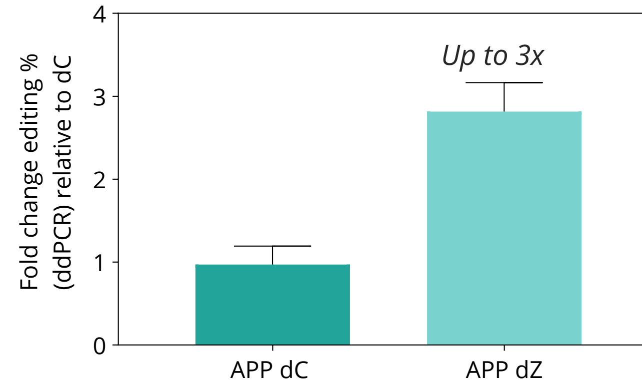
Editing of WT *APP* RNA in human ARPE-19

Transfection of 100nM EON, N=3, 48 hours



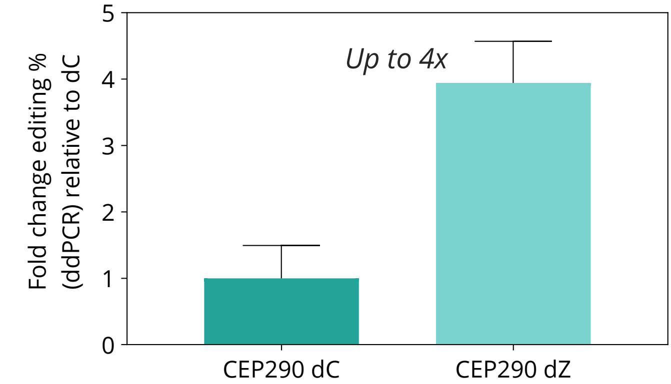
Editing of *APP* WT RNA in human retinal organoids

Gymnosis, 10μM single dose + 40μM CQ, N=6, 4 weeks



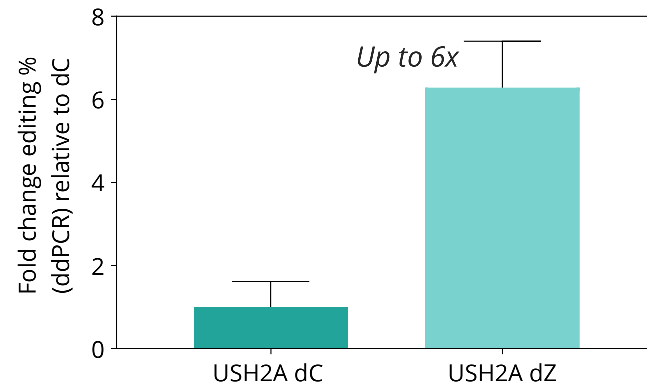
Editing of *hCEP290* K1575X in human LCA retinal organoids

Gymnosis, 10μM single dose, N=8, 4 weeks



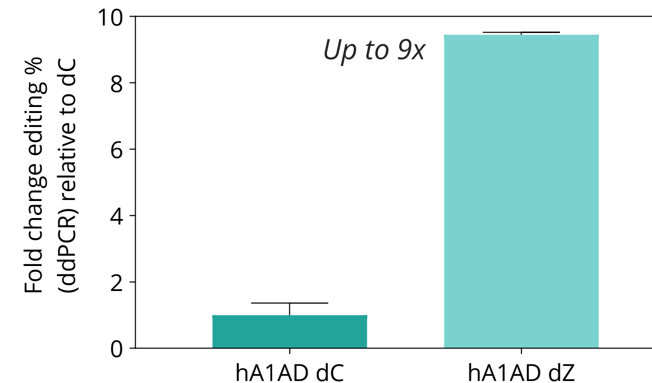
Editing of *USH2A* WT RNA in human retinal organoids

Gymnosis, 15μM single dose + 40μM CQ, N=4, 4 weeks



Editing of *SERPINA1* E366K in A1AD patient hepatocytes

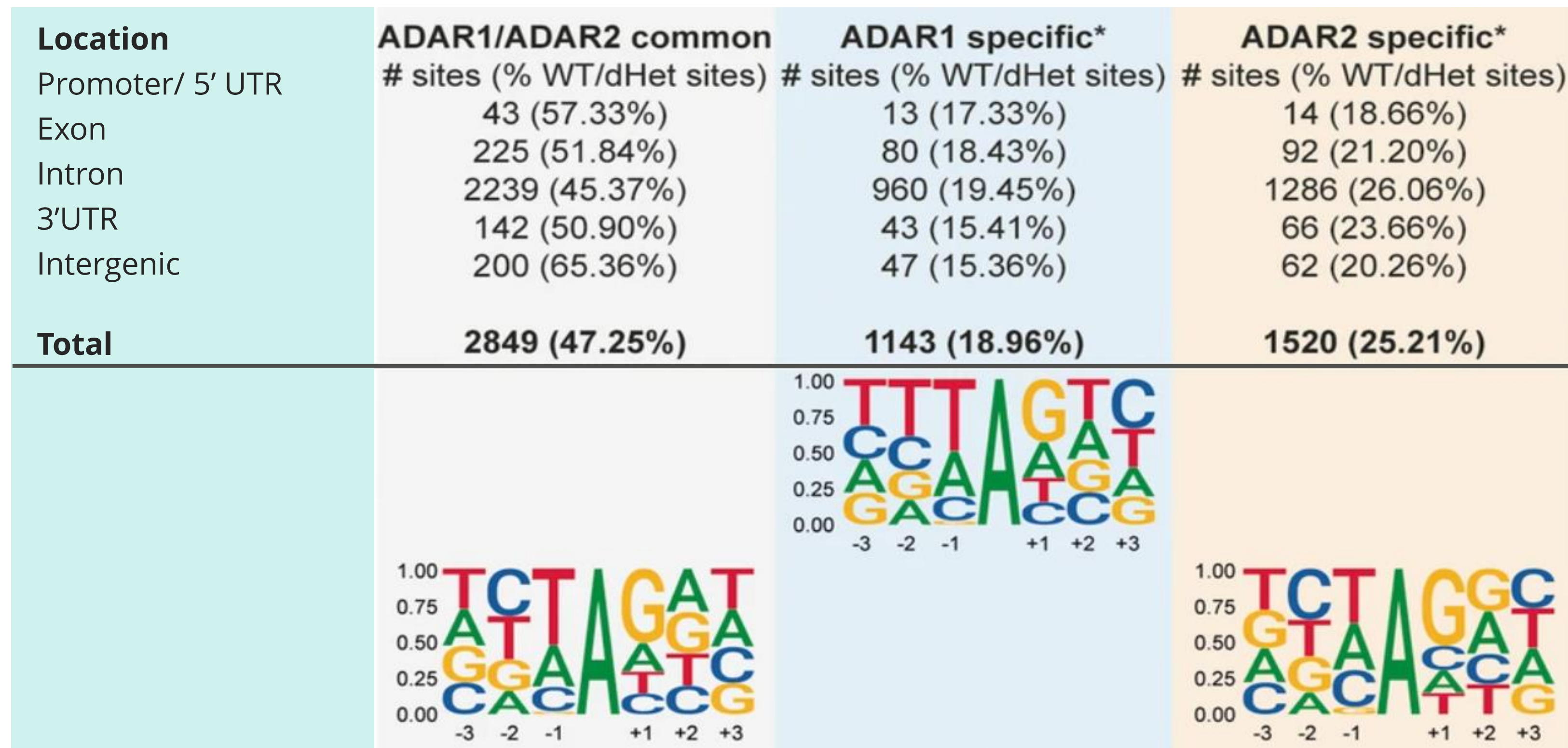
Transfection of 100nM EON, N=2, 48 hours



ADAR knows few sequence constraints

With the exception of G upstream of target adenosine (5'-GA-3')

This has wide implications for the applicability of targeted RNA editing – guide RNAs with Watson-Crick complementarity are enough to recruit ADAR and induce targeted editing

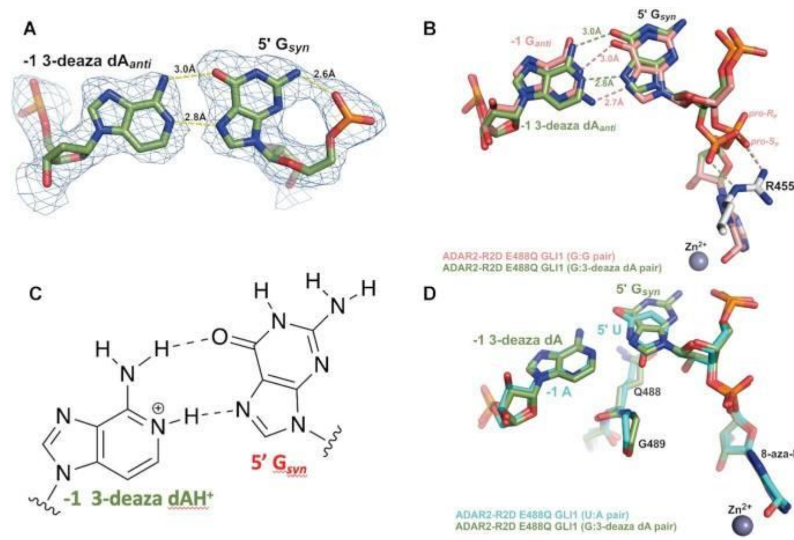


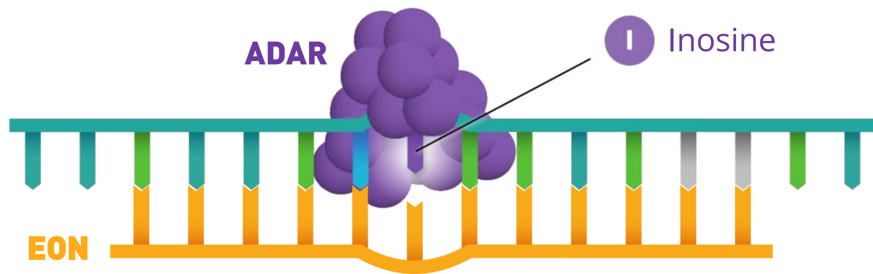
Location	ADAR1/ADAR2 common # sites (% WT/dHet sites)	ADAR1 specific* # sites (% WT/dHet sites)	ADAR2 specific* # sites (% WT/dHet sites)
Promoter/ 5' UTR	43 (57.33%)	13 (17.33%)	14 (18.66%)
Exon	225 (51.84%)	80 (18.43%)	92 (21.20%)
Intron	2239 (45.37%)	960 (19.45%)	1286 (26.06%)
3'UTR	142 (50.90%)	43 (15.41%)	66 (23.66%)
Intergenic	200 (65.36%)	47 (15.36%)	62 (20.26%)
Total	**2849 (47.25%)**	**1143 (18.96%)**	**1520 (25.21%)**

Adapted from Eggington et al. Predicting sites of ADAR editing in double-stranded RNA. Nat Commun. 2011;2:319

A single base change opposite the target 5’G greatly enhances editing

PROQR – UC DAVIS COLLABORATION

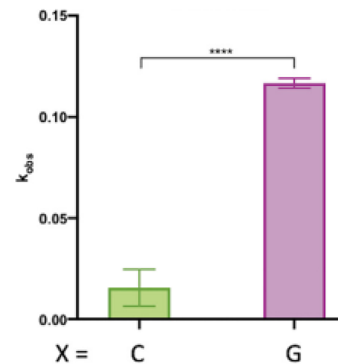


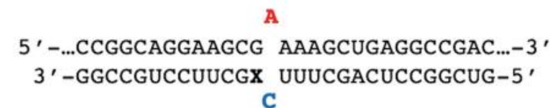


3-deaza-dA in EER

In vitro deamination kinetics for ADAR2 and duplex RNAs derived from WT *hMECP2*

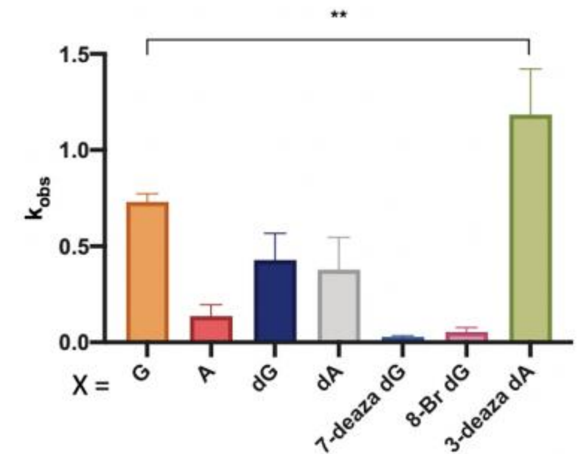
100 nM ADAR2, 3 technical replicates, mean, SD

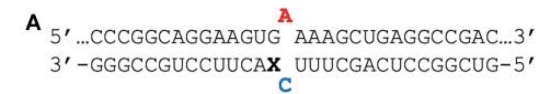




In vitro deamination kinetics for ADAR2 and duplex RNAs derived from *hMECP2* R255X

100nM ADAR2, 3 technical replicates, mean, SD





Adapted from Doherty EE, et al. Nucleic Acids Res. 2022;50(19):10857-10868.

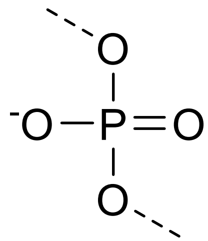
Statistical significance between groups was determined using one-way ANOVA with Tukey’s multiple comparisons test or an unpaired t-test with Welch’s correction; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Modification in the ADAR-binding region (ABR)

Examples of structure–activity relationship (SAR) assessment interrogating the impact of neutral linkage modifications

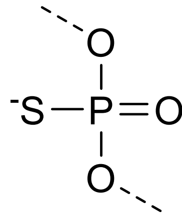
Different linkage modifications commonly encountered in oligo therapeutics

PO



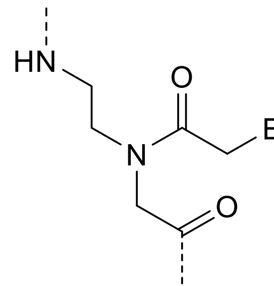
Low stability

PS

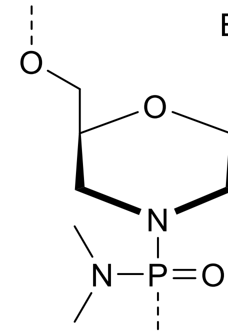


+ Increases cellular uptake and improves t1/2 *in vivo* by virtue of increased protein binding. Tolerability observations

PNA

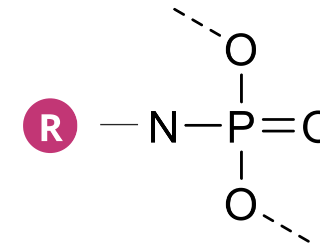


PMO

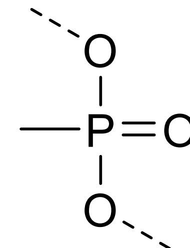


several RNA-modulating applications, challenge for A→I editing due to certain enzyme contacts being required for ADAR recognition

PN



PMe

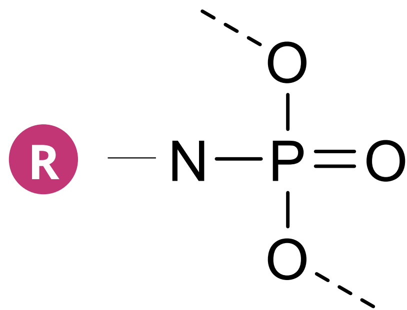


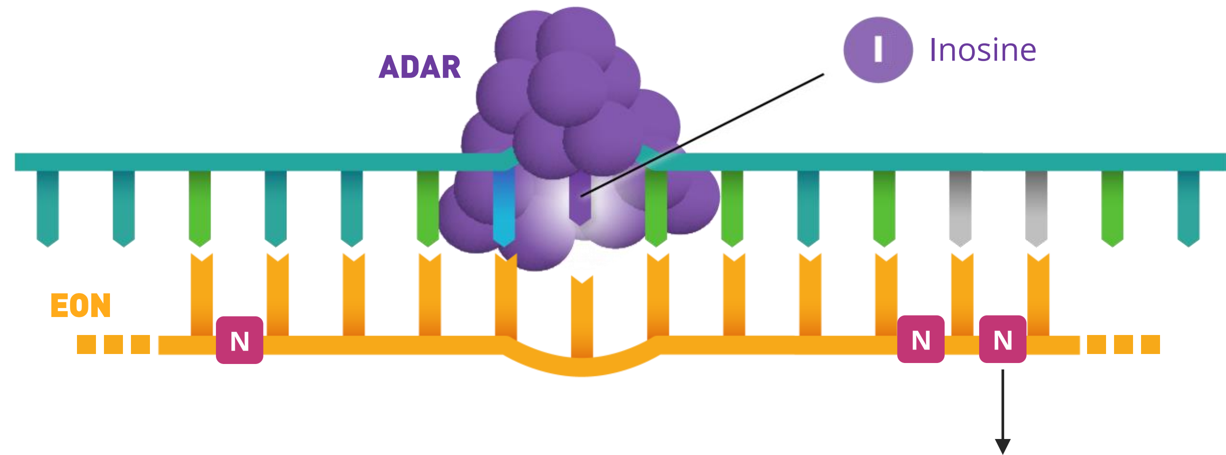
Neutral linkages
Decreased nuclease degradation,
Remove all PS

Effect of phosphoroamidate linkage on EONs editing activity in different models

Phosphoroamidate linkage

PN

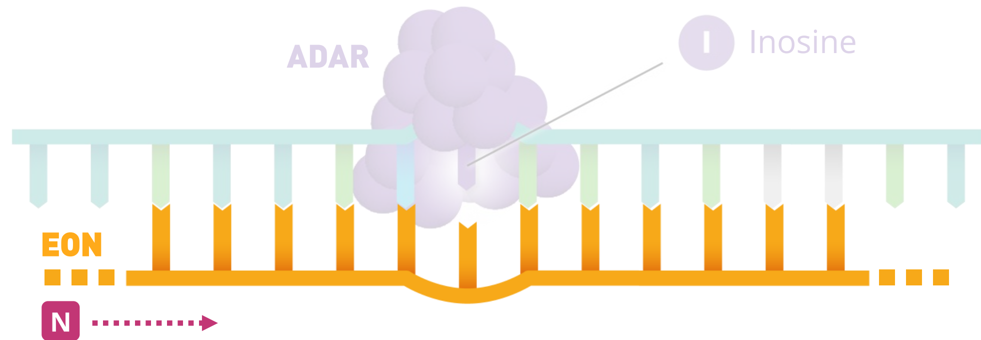




Phosphoroamidate linkage modifications in the ABR

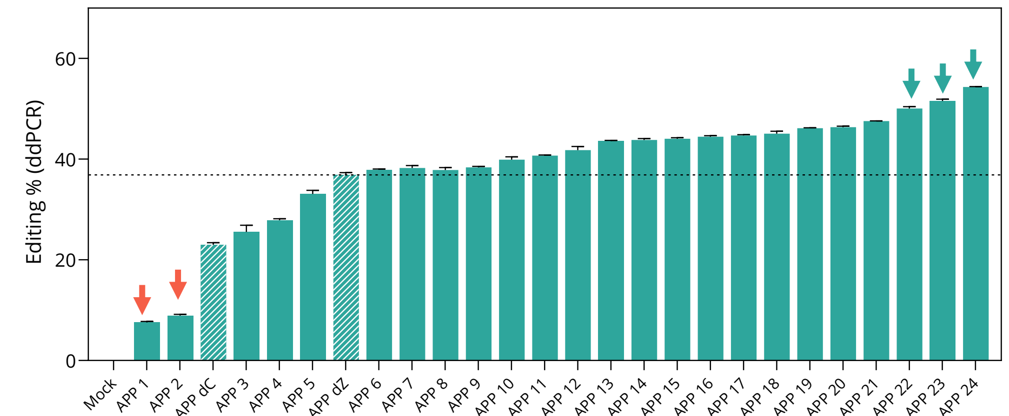
To enhance metabolic stability and activity

Introduction of PN to EON showing the critical impact on editing efficiency



RNA editing of WT *APP* in human ARPE-19

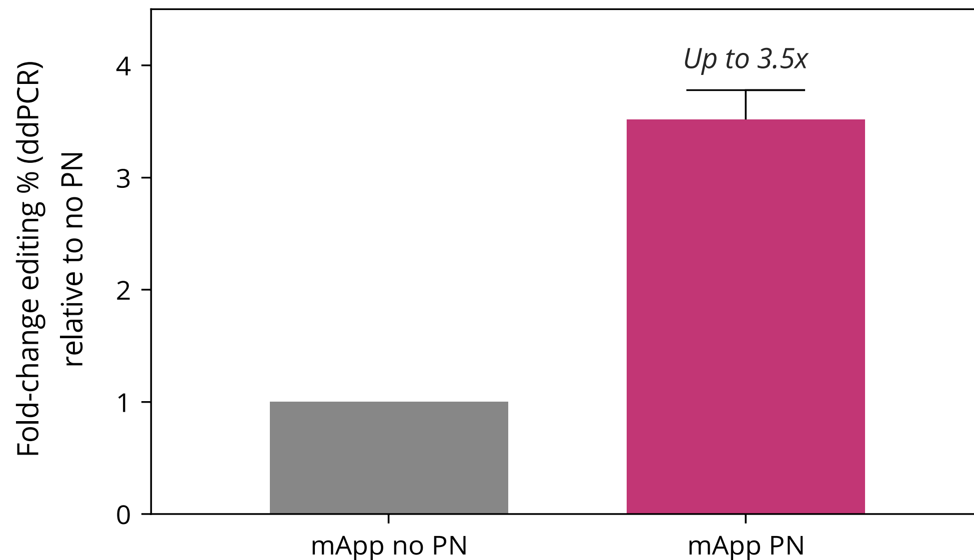
Transfection, N=2, 2 days, 100nM, ddPCR, Mean, SD

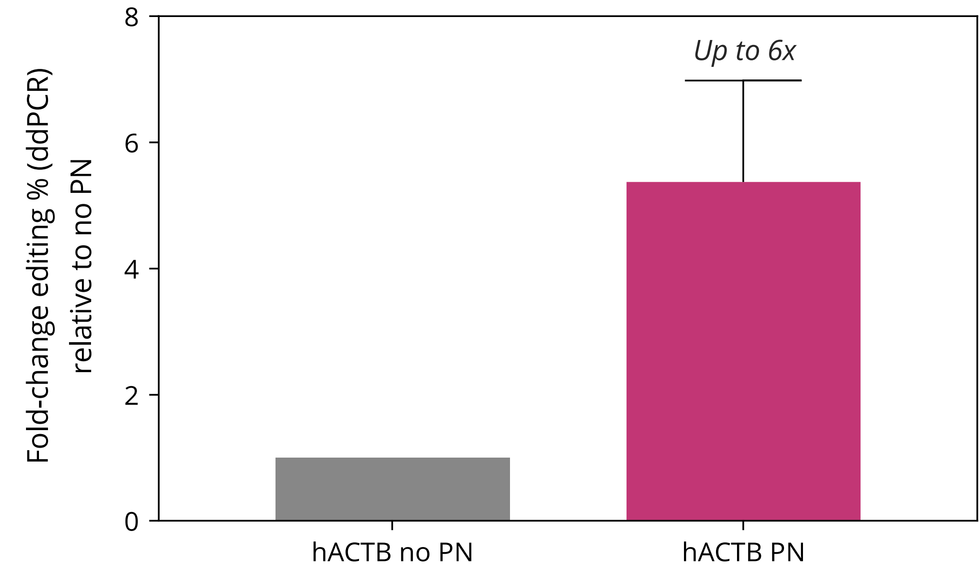


EON	Structure
APP dC – No PN, dC base	$A_XU_XC_WA_XC_XU_XG_XU_XC_XG_ZC_X\mathrm{dC}A_XU_YG_XA_XC_ZA_XA_XC_WA_XC_XC_XG_XC$
APP dZ – No PN, dZ base	$A_XU_XC_WA_XC_XU_XG_XU_XC_XG_ZC_X\mathbf{dZ}A_XU_YG_XA_XC_ZA_XA_XC_WA_XC_XC_XG_XC$
APP 1-24 – dZ and PN at different positions	$A_{N}...U_XC_WA_XC_XU_XG_XU_XC_XG_ZC_X\mathbf{dZ}A_XU_YG_XA_XC_ZA_XA_XC_WA_XC_XC_XG_XC$

- The sequences contain a mix of 2’-*O*-Me, DNA, PMe, PS, 2’-F, 2’-MOE
- The changing factor is +/- dZ in EER and +/- PN (N) with systematic change in location
- Each letter coding shows a combination of linkage and sugar modifications
- PN increases EON editing up to 1.5x and, in some positions, have negative effect on editing

Effect of PN linkages on EONs editing activity in different models



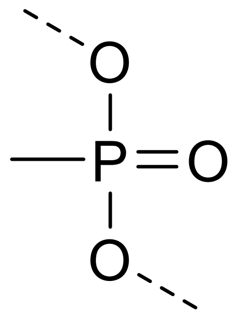


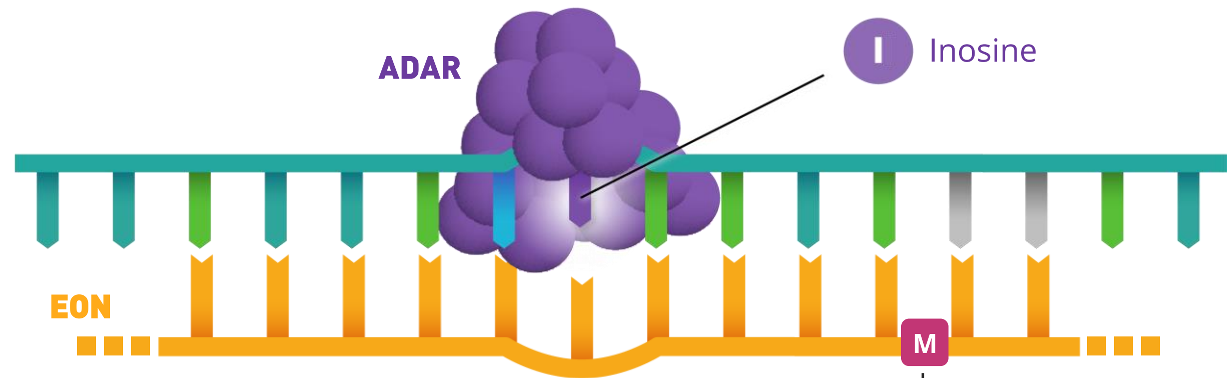
- The sequences contain a mix of 2'-O-Me, DNA, PMe, PS, 2'-F, 2'-MOE
- The changing factor is +/- 2 PNs at the same locations

PMe linkages on EONs editing activity in different models

Methylphosphonate linkage

PMe



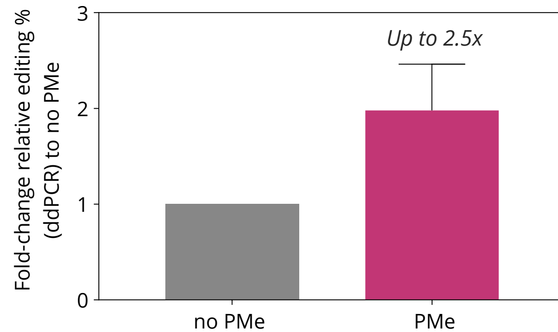


PMe phosphate linkage modifications

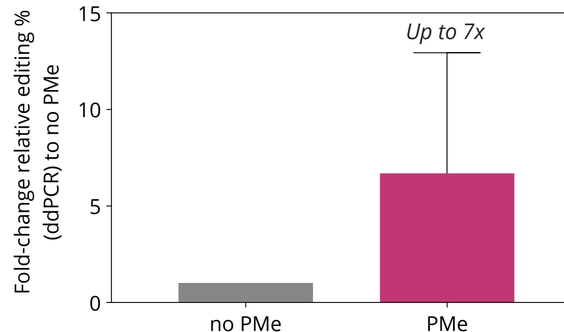
To enhance metabolic stability and activity

Effect of PMe linkages on EONs editing activity in different systems

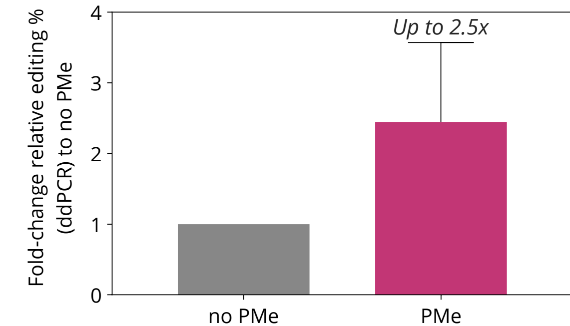
RNA editing of m*App in mRPE cells*
Gymnosis, 5μM, single dose, N=4, 5 days, ddPCR, mean, SEM



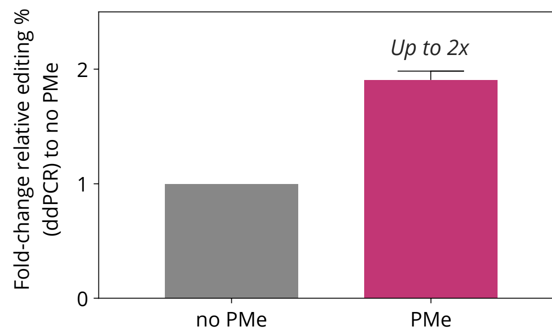
RNA editing of h*APP in ARPE-19 cells*
Gymnosis, 5μM, single dose, N=2, 5 days, dPCR, mean, SEM



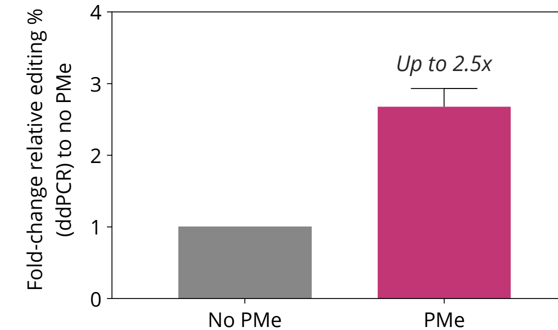
RNA editing of h*APP in retinal organoids*
Gymnosis, 5μM, single dose, N=6, 14 days, ddPCR, mean, SEM



RNA editing of *hAPP* in Weri-rb1 cells
Gymnosis, 5μM single dose, N=2, 5 days, dPCR, mean, SEM

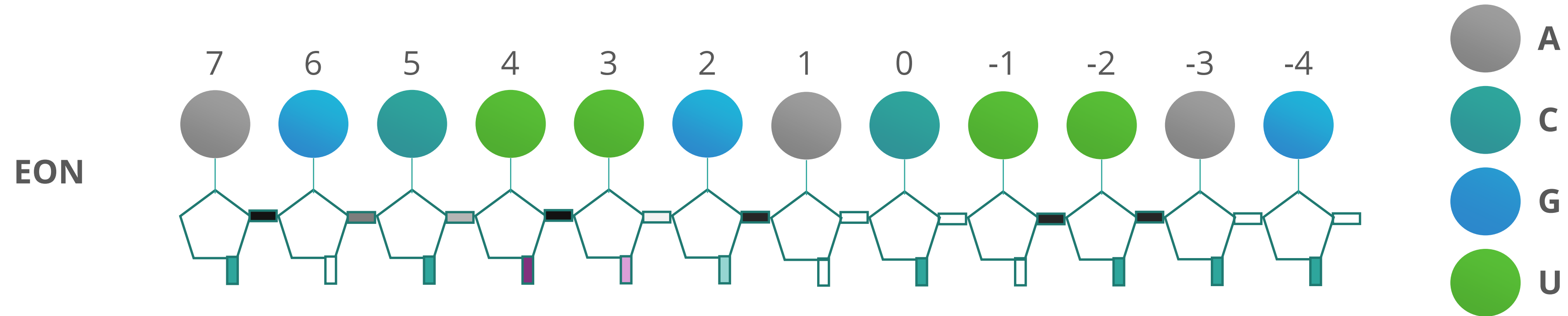


RNA editing of *hAPP* in HepG2 cells
Gymnosis, 5μM single dose, N=2, 5 days, dPCR, mean, SEM



The sequences contain a mix of 2'-O-Me, DNA, PN, PS, 2'-F, 2'-MOE and the changing factor is +/- 1 PMe at the same location

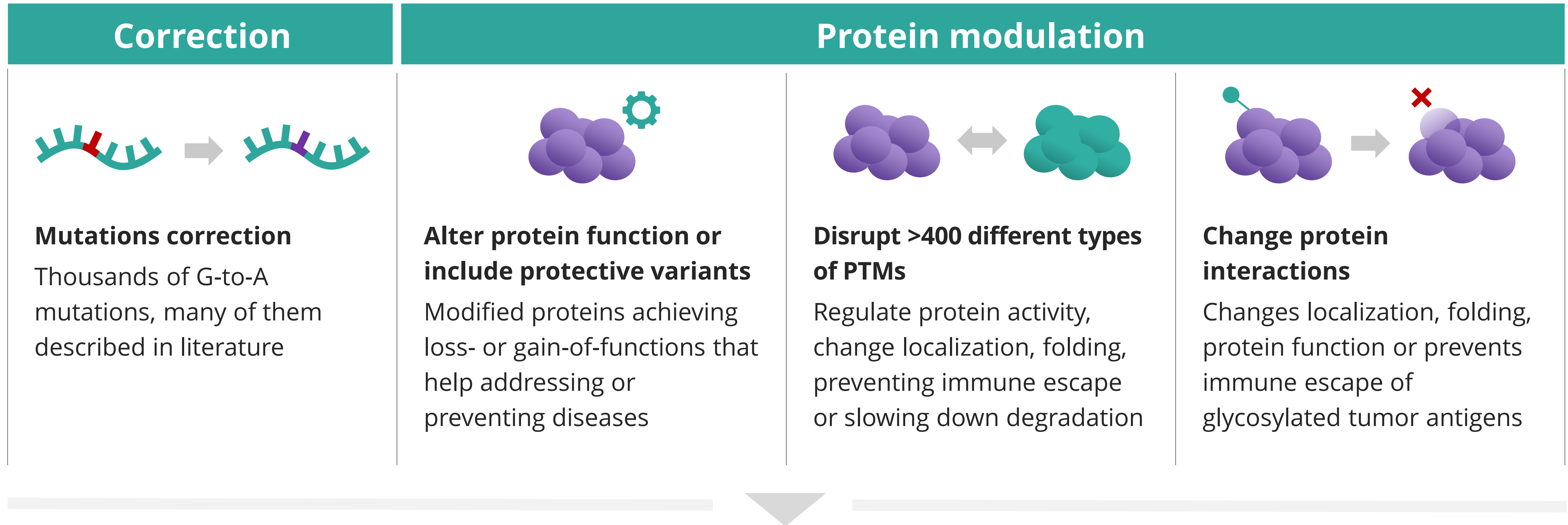
Accelerating program advancement with focus on design principles



Aspect	Determined by	Modifications	Effects
Base	Target RNA	Mismatches and analogs (**dZ**)	Improved PD
Ribose modification	ADAR structure	2′-H, 2′-*O*-Me, 2′-MOE, 2′-F, 2′-NH_2, LNA, TNA, UNA, 2′,2′-diF, FANA	Improved PK and PD
Linkage	ADAR structure	PO; PS; **PN**; **PMe**; PAc	Improved PK and PD

ADAR: Adenosine deaminase acting on RNA, EON: Editing oligonucleotide, PD: pharmacodynamic, PK: pharmacokinetic

Axiomer® creating a new class of medicines with broad therapeutic potential

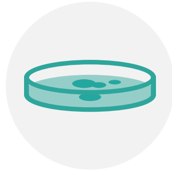


BROAD THERAPEUTIC POTENTIAL

- Common diseases
- Rare diseases
- Target a wide variety of organs
- Treat so-far undruggable targets

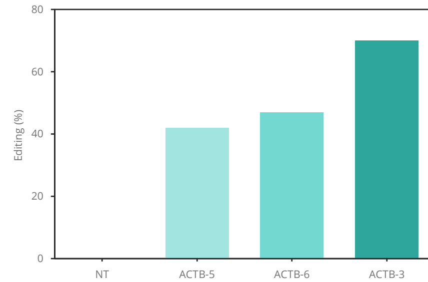
PTMs: Post-translational modifications.

Advancing Axiomer® development across different models and targets in the liver

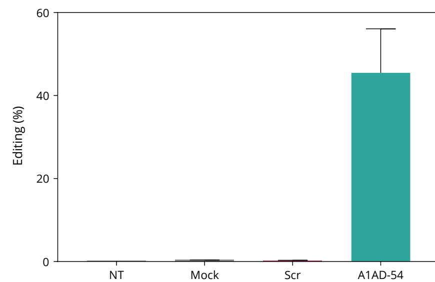


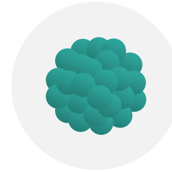
Cell models

Up to 70% RNA editing of *ACTB* in human primary hepatocytes



>50% RNA editing of *SERPINA1* E366K in human A1AD patient hepatocytes

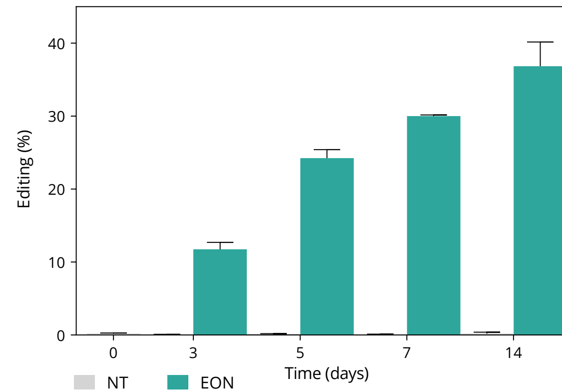




Liver organoids

Up to 40% RNA editing of *ACTB* in human LMTs

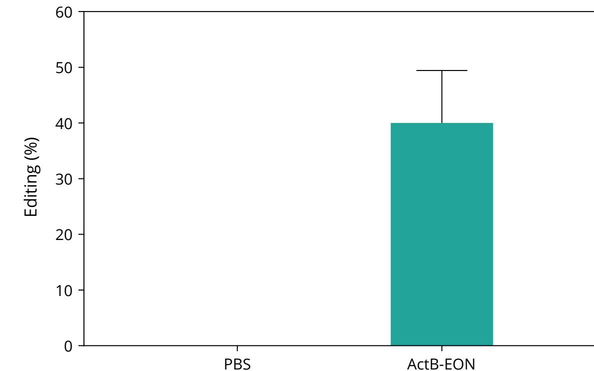
Gymnosis, 1µM, constant dose, 3 pools of 24 LMTs per condition, 14 days, dPCR, mean, SD



Mice *in vivo*

Up to 50% RNA editing of *ActB* in liver of mice

SC, 5 daily doses of 10 mg/kg, N=4, 1 week, ddPCR, mean, SD



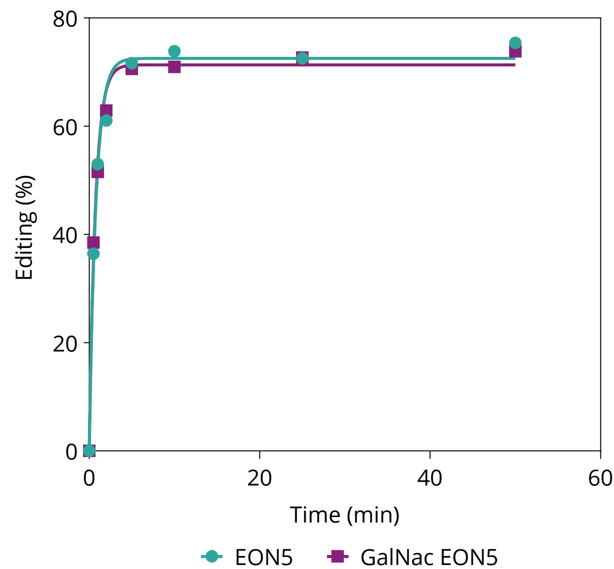
Conditions of *ACTB* editing experiment in human primary hepatocytes experiment: gymnosis, 10µM, single dose, N=1, 48 hours, dPCR; Conditions of the of *SERPINA1* editing experiment in human A1AD patient hepatocytes experiment: transfection, 100 nM, single dose, N=2, 47 hours, dPCR, mean, SD. LMTs: human liver microtissues.

GalNAc increases RNA editing efficiency

BEA assay

GalNAc does not interfere A-to-I editing *in vitro*

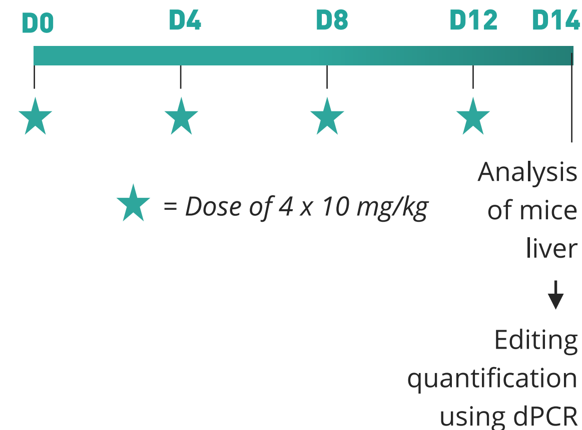
*2nM target RNA, 6nM EON and 6nM ADAR2, N=1, BEA assay**



Mice *in vivo*

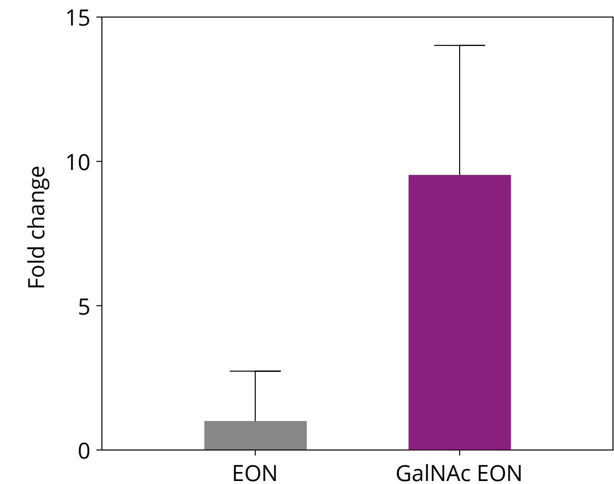
Mice treatment

In vivo



10-fold change in editing in liver of mice**

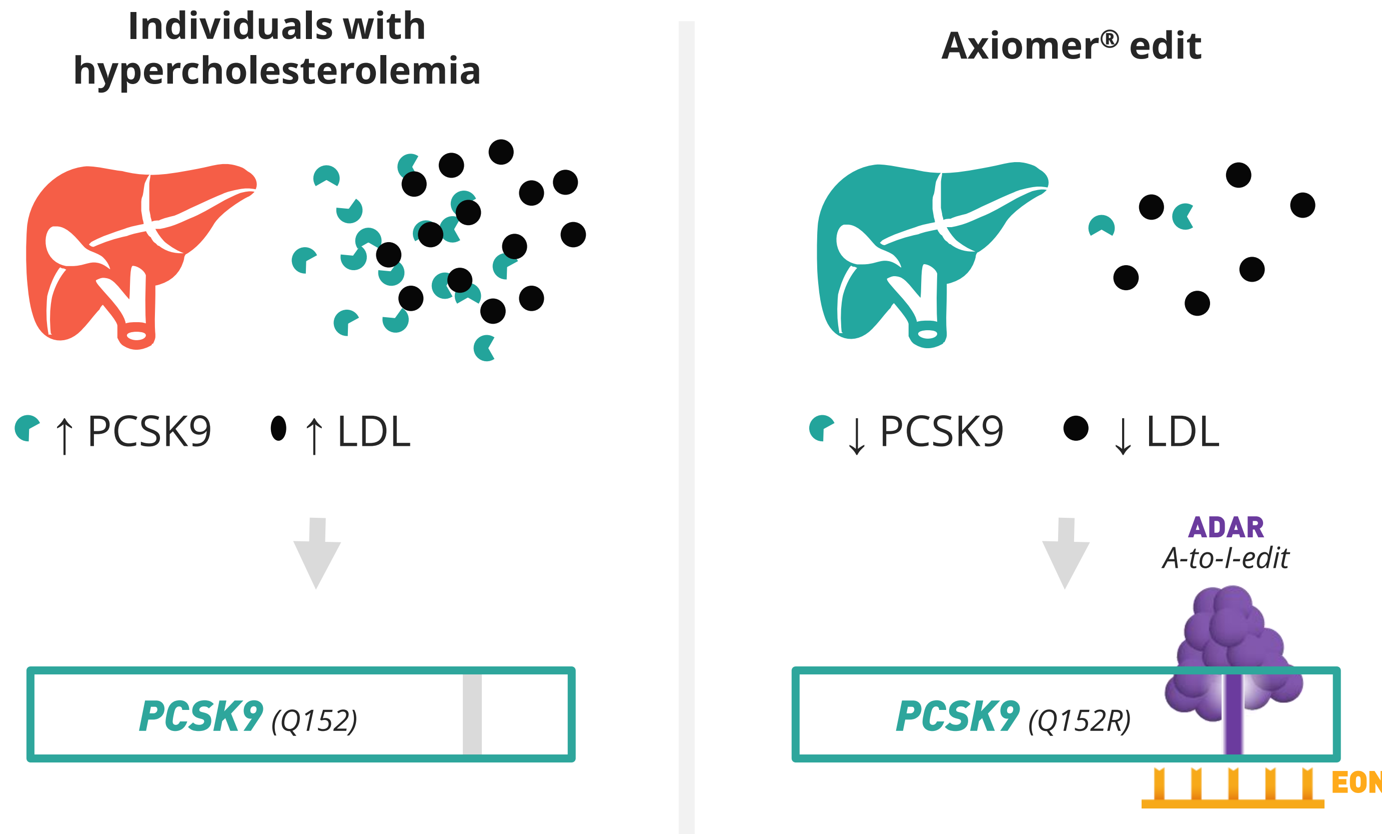
SC, 4 doses of 10 mg/kg, N=4-5, 2weeks, dPCR, mean, SD



BEA, Biochemical editing assay; SC, subcutaneous; SD, standard deviation. *BEA assay timepoints 0, 0.5-, 1-, 2-, 5-, 10-, 25- and 50-min. **Undisclosed target.

Changing the autocleavage site with Axiomer® leads to a LOF in PCSK9

Generation of a loss-of-function variant to lower PCSK9

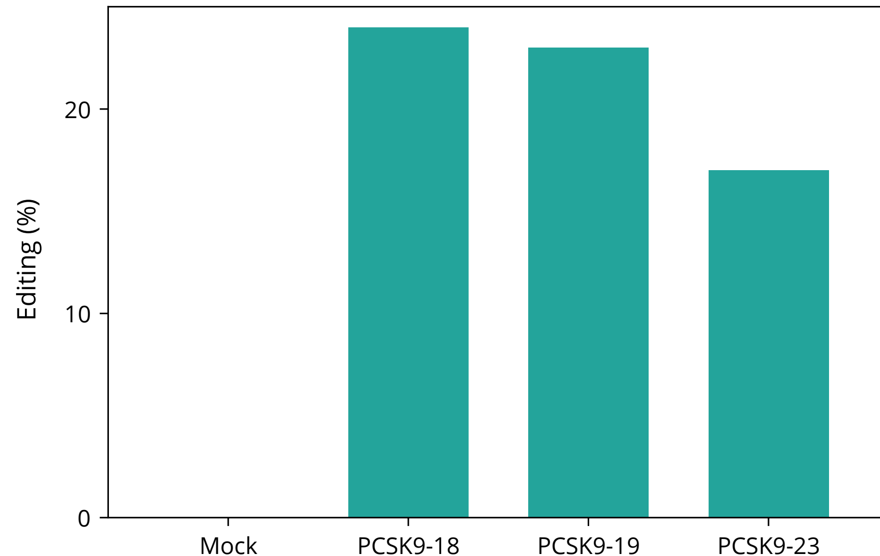


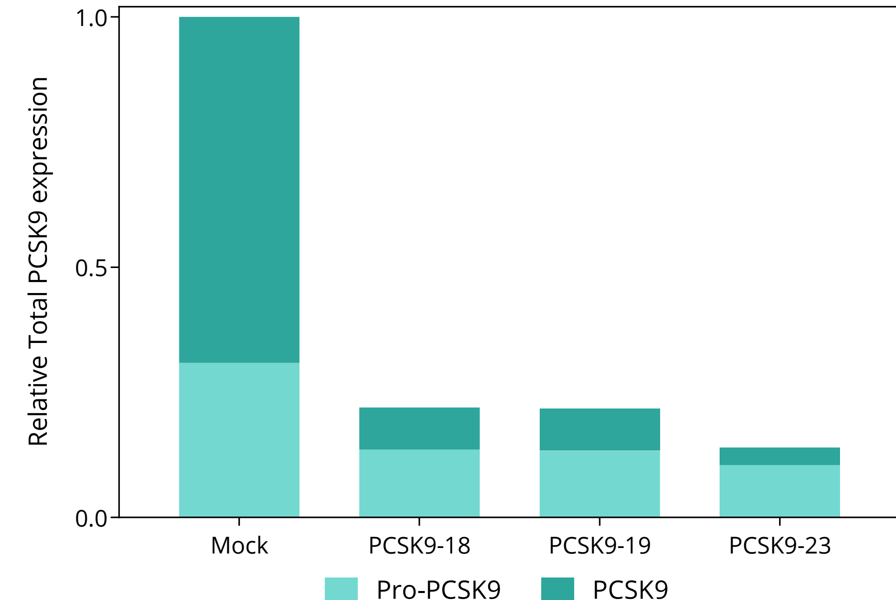
Disruption of PCSK9 autocleavage site reduces protein in bloodstream

- Less PCSK9 leads to increase of LDL-R on cells, decrease of 'bad' LDL in bloodstream
- Loss-of-function *PCSK9* variant Q152H is associated with low plasma LDL cholesterol in a French-Canadian family and with impaired processing and secretion in cell culture

LDL: Low density lipoprotein, LDL-R: Low density lipoprotein receptor. LOF: Loss of function. Reference: Mayne J, et al. Clin Chem. 2011 Oct;57(10):1415-23.

Editing of *PCSK9* RNA results in a proenzyme with dominant negative properties



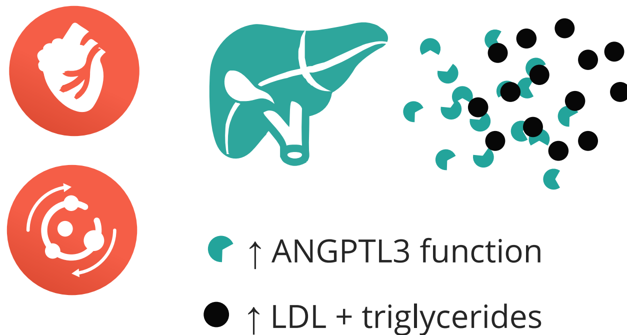


- Up to 25% A-to-I editing of *PCSK9* RNA detected using ddPCR assays leading up to 80% reduction of total PCSK9 protein
- Shift in the ratio cleaved to uncleaved PCSK9 observed; 70%:30% in mock to 25%:75% in treated samples
- The inability to undergo autocleavage likely retains the proenzyme in the endoplasmic reticulum where it can act as a dominant negative protein, preventing the exit of the wild-type form of PCSK9.

Changing a protein binding site in ANGPLT3 with Axiomer® leads to LPL activation

Generation of a variant to inhibit heparin binding

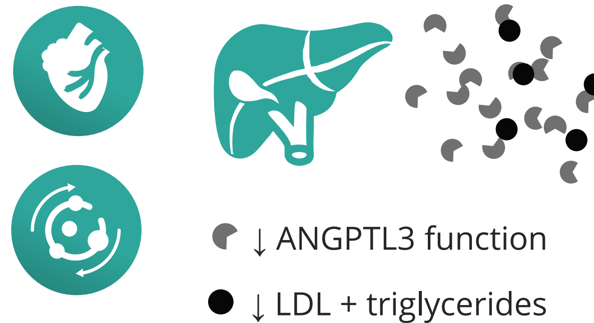
Individuals with increased CVD risk



↑ ANGPTL3 function

↑ LDL + triglycerides

Axiomer® edit



↓ ANGPTL3 function

↓ LDL + triglycerides

Wildtype ANGPTL3

AAAGACTTTGTCCAT**AAG**ACGAAGGGCCAAATTAAT
-K--D--F--V--H--**K**--T--K--G--Q--I--N-

Edited ANGPTL3

AAAGACTTTGTCCAT**GAG**ACGAAGGGCCAAATTAAT
-K--D--F--V--H--**E**--T--K--G--Q--I--N-

= Heparin-binding motif

ANGPTL3 is an angiopoietin-like factor that inhibits lipoprotein lipases (LPL)

- Increase triglyceride, cholesterol, and non-esterified fatty acids in plasma leading to an increased risk of CVD

Reported variant of ANGPTL3

- Significantly decreased triglycerides, LDL-cholesterol, and total cholesterol
- Significantly decreased odds ratio for coronary artery disease

Heparin binding was shown to be essential for proper ANGPTL3 function

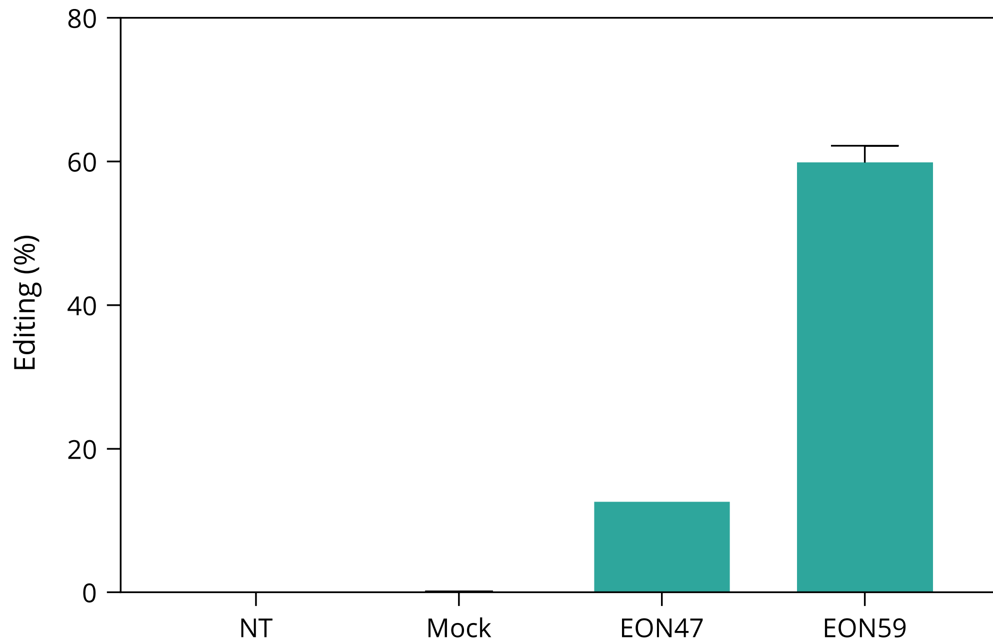
- Disruption of the heparin binding site is highly likely to abrogate LPL inhibition, ultimately leading to lipid lowering in the serum

CVD; cardiovascular disease. LDL: low density lipoprotein, LOF: Loss of function. References: Ono M et al. J Biol Chem. 2003 Oct 24;278(43):41804-9; Romeo S et al. J Clin Invest. 2009 Jan;119(1):70-9; Dewey FE et al. N Engl J Med. 2017 Jul 20;377(3):211-221.

ANGPTL3 variant disrupting essential protein binding site

More than 60% RNA editing of *ANGPTL3* in primary human hepatocytes derived spheroids

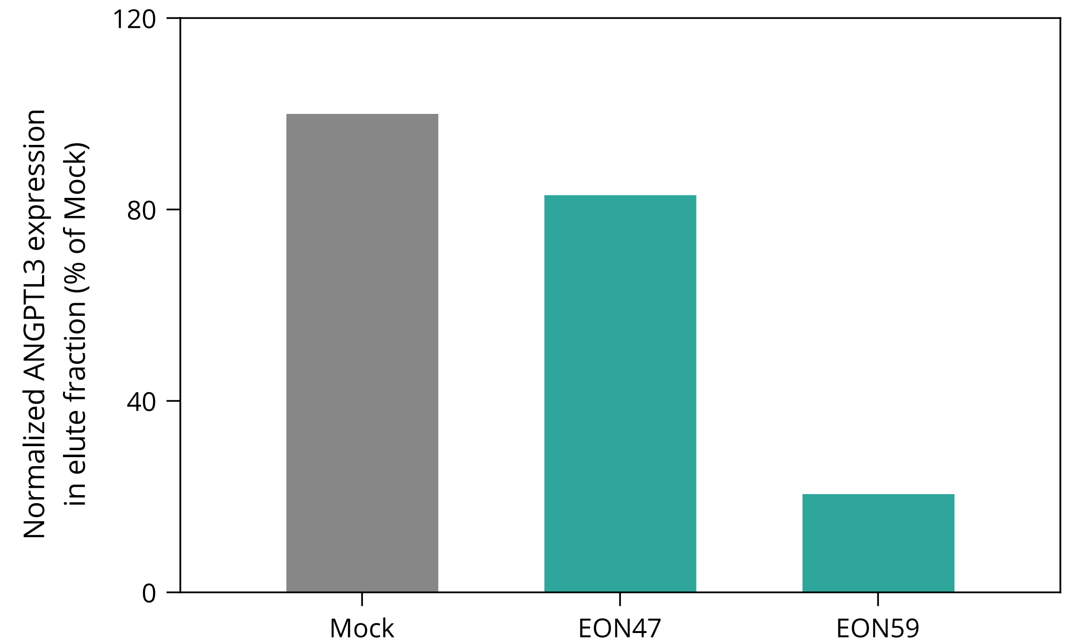
Gymnosis, 1μM, single dose, N=1 or 2, 5 days, dPCR, mean, SD



More than 60% RNA editing of *ANGPTL3* in primary human hepatocytes derived spheroids

Up to 80% decrease in heparin binding in Huh-7 cells

Gymnosis, 1μM, single dose, N=1, 72 hours, western blot



Up to 80% decrease in heparin binding in Huh-7 cells

ProQR leading research to optimize EONs for therapeutic use

Modification of the orphan base

in the EER confirm superiority of dZ base

Collaborative work to address 5’GA context

and broaden EONs therapeutic potential

Structure–activity relationship (SAR) assessment

to define guiding principles

Positive impact of neutral linkage modifications

in the ABR (PN, PMe)

Further improvements led to approx. 50% editing

in liver mice

New optimizations combined for pipeline development

targeting liver originated disorders

IT'S IN
OUR RNA