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RNA Editing: Expanding the Potential of RNA Therapeutics

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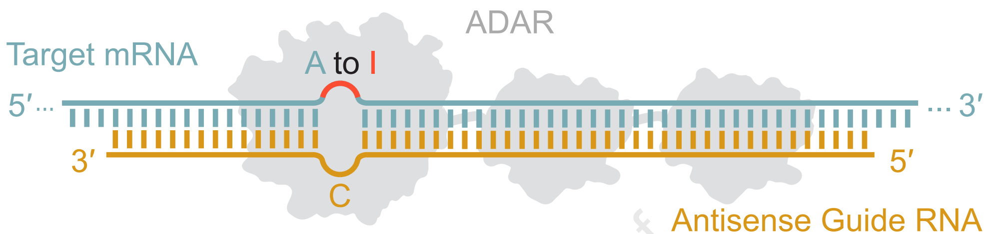
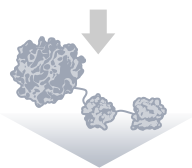
Therapeutic A-to-I RNA Editing



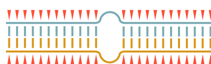
Cellular Editing Environment



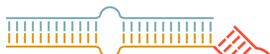
ADAR Isoform Expression and Regulation

gRNA Design & Optimization

Abundance & Stability



Structure & Composition

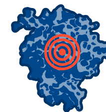


Recruitment

Specificity

Translation to the Clinic

Delivery & Biodistribution



Molecular Mechanisms & Therapeutic Opportunities

Saftey & Toxicity

Clinical Outcome

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3 **RNA Editing: Expanding the Potential of RNA Therapeutics**
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17 **ABSTRACT**

18 RNA therapeutics have had a tremendous impact on medicine, recently exemplified by the rapid
19 development and deployment of mRNA vaccines to combat the COVID-19 pandemic. In
20 addition, RNA-targeting drugs have been developed for diseases with significant unmet medical
21 needs through selective mRNA knockdown or modulation of pre-mRNA splicing. Recently,
22 RNA editing, particularly antisense RNA-guided Adenosine Deaminase Acting on RNA
23 (ADAR) based programmable A-to-I editing, has emerged as a powerful tool to manipulate RNA
24 to enable correction of disease-causing mutations and modulate gene expression and protein
25 function. Beyond correcting pathogenic mutations, the technology is particularly well suited for
26 therapeutic applications that require a transient pharmacodynamic effect, such as the treatment of
27 acute pain, obesity, viral infection, and inflammation, where it would be undesirable to introduce
28 permanent alterations to the genome. Furthermore, transient modulation of protein function, such
29 as altering the active sites of enzymes or the interface of protein-protein interactions, opens the
30 door to therapeutic avenues ranging from regenerative medicine to oncology. These emerging
31 RNA editing-based toolsets are poised to broadly impact biotechnology and therapeutic
32 applications. Here, we review the emerging field of therapeutic RNA editing, highlight recent
33 laboratory advancements, and discuss the key challenges on the path to clinical development.

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36 **INTRODUCTION**

37 Large-scale genome sequencing has progressively revealed the causal genetic variation
38 underlying many human diseases.[1, 2] This information has driven significant innovation in
39 biotechnology and ushered in the modern era of DNA and RNA therapeutics. While DNA
40 targeting can result in durable and potentially permanent cures, RNA targeting modalities enable
41 tunability and reversibility. The lack of permanent off-targets offers unique advantages in
42 specific therapeutic settings. Here we focus on recently emerging precision RNA editing
43 approaches, especially those based on Adenosine Deaminases Acting on RNA (ADARs) that are
44 enabling programmable endogenous RNA modulation beyond RNA knockdown or
45 overexpression.

46

47 ADARs represent a family of enzymes that deaminates RNA adenosines (A) into inosines (I)
48 within double stranded RNA (dsRNA). Inosine is functionally recognized by the cellular
49 machineries as guanosine (G), thereby allowing the enzyme to modulate translation, splicing, or
50 any regulatory mechanism reliant upon an adenosine containing motif. A-to-I RNA editing was
51 discovered in the late 1980s[3, 4] and a proposal to leverage ADARs for therapeutic purposes was
52 first proposed in 1995.[5] Over the past decade, there has been a renewed interest in the
53 development of this RNA targeting modality, with numerous groups demonstrating the
54 redirection of endogenous ADAR activity for site-specific A>G editing using guide RNAs
55 (gRNA) antisense to a target messenger RNA of interest in human cells and *in vivo* animal
56 models,[6-14] as well delivery of exogenous ADARs to enable targeted RNA editing.[15, 16]

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59

60 Notably, G-to-A missense and nonsense mutations account for twenty-eight percent of
61 pathogenic single nucleotide variants (SNVs) reported on ClinVar
62 (https://www.ncbi.nlm.nih.gov/clinvar/ accessed on April 13, 2022) and can be targeted for
63 ADAR-mediated restoration of the wild-type sequence. More broadly, adenosines are critical for
64 many functional sites within RNA, such as translation initiation sites (TIS), splice acceptor and
65 donor sites, microRNA binding sites, and polyadenylation signals (PAS). This further expands
66 the therapeutic potential for RNA editing to regulate protein expression levels and splicing and
67 may be additive with current approaches that utilize ASOs for masking TIS, splice sites, or
68 polyadenylation signals. Furthermore, A-to-G changes can result in 17 different amino acid
69 substitutions, enabling the modulation of protein function and protein-protein interactions.
70 Indeed, natural ADAR function has been shown to modulate proteins with 55 editing sites
71 identified in coding regions,[17] many of which are conserved across species.[18] Nonsense
72 mutations (UAG, UGA, UAA) can be recoded to a tryptophan (UGG), which may be tolerable to
73 a protein, depending on the exact position of the nonsense mutation.[19, 20] Altogether, RNA
74 editing opens a wide range of opportunities for therapeutic and protective benefits to patients.
75 Correspondingly there is a growing interest in clinical translation, with many academic labs and
76 biotechnology companies now focused on refining and tuning this technology with a goal of
77 enabling human therapeutic applications.

78

79 Several challenges, however, must be overcome to bring the therapeutic potential of RNA
80 editing to patients. ADAR is inherently promiscuous and has the potential to deaminate any
81 adenosine within a dsRNA structure. Thus, gRNA-directed RNA editing has the potential for

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82 bystander and off-target editing, as well as possible unintended impact on splicing and
83 translation. Furthermore, ADAR has natural sequence preferences that may not align with a
84 chosen therapeutically relevant adenosine. These challenges highlight the need for exquisite
85 gRNA engineering that enables highly efficient and specific RNA editing. Additionally, non-
86 clinical and clinical assays to quantify editing efficiency and transcriptome integrity are
87 necessary to establish safety metrics to support clinical development.

88

89 Beyond RNA editing-specific challenges, issues of delivery and manufacturing that broadly
90 impact the fields of gene therapy and antisense oligonucleotide therapy must also be addressed.
91 For example, while delivering payloads with an Adeno-associated virus (AAV) vector has
92 significant clinical precedent, issues persist related to manufacturing, quality control, and safety,
93 while the possibility for immunogenicity and transgene silencing may hinder efficacy.
94 Furthermore, the narrow tropism of wild-type AAVs and biodistribution of ASOs limits delivery
95 to the liver, muscle, and direct injection into the central nervous system (CNS), while ASOs are
96 also readily absorbed in the kidney[21]. Solutions to each of these stated challenges are in
97 development as the field of RNA editing advances towards the clinic. We will first review the
98 underlying biology of ADAR-mediated RNA editing and how it can inform its therapeutic
99 application.

100 FACTORS THAT AFFECT ENDOGENOUS RNA EDITING

101 Since the discovery of ADAR in 1987,[3, 4] much progress has been made in understanding the
102 natural biological functions of this enzyme group. Understanding fundamental ADAR biology,
103 including various isoforms and structures, expression and regulation, and cellular and subcellular
104 localization, is critical to unlocking the therapeutic potential of RNA editing. Thus, we begin by

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105 reviewing key aspects of ADAR biology that can inform drug design, development, and translation
106 to the clinical. Key variables that impact A-to-I RNA editing are also depicted in **Figure 1.**

107 **ADAR isoforms and structure**

108 The ADAR family is composed of three genes that encode five different protein isoforms:
109 ADAR1p110, ADAR1p150, ADAR2a, ADAR2b, and ADAR3. Each isoform contains N-
110 terminal double stranded RNA binding domains (dsRBDs) followed by a C-terminal deaminase
111 domain. All isoforms possess a nuclear localization signal (NLS), while ADARp150 also has a
112 nuclear export signal (NES) that promotes cytosolic localization. ADAR2 is spliced in several
113 isoforms, with only ADAR2a and ADAR2b being translated into proteins. ADAR2b contains an
114 Alu insertion in the deaminase domain, which may explain the 50% reduction in activity
115 compared to ADAR2a.[22] ADAR3 lacks deaminase activity but may play a role in regulating
116 RNA editing through competitive antagonism with ADAR1 and ADAR2.[23-25] The structural
117 differences between ADAR isoforms are responsible for subtle differences in their substrate
118 preferences that must be taken into consideration during the gRNA design process to ensure
119 efficient and selective editing depending on the isoform present in the tissue and cell type of
120 interest.
121

122 The substrate preferences of ADAR can be mechanistically traced back to its structure. The
123 dsRBDs engage a 12 - 14 bp stretch of dsRNA with specificity to the A-form helix and ribose 2'
124 hydroxyl groups that distinguish it from dsDNA.[26] The shallow minor groove of the A-form
125 helix provides access to the bases and allows for sequence-specific contacts, which can explain
126 how dsRBDs from various proteins have unique binding preferences. Indeed, ADAR dsRBD
127 binding selectivity has been shown to influence editing selectivity,[27] and replacing the dsRBDs

128 of ADAR1 with PKR significantly alters the editing activity.[28] One of the most well-studied
129 ADAR substrates is the GRIA2 R/G site, which forms an evolutionarily conserved hairpin
130 structure driven by hybridization of exon 13 to the downstream intron and contains three
131 mismatches within the RNA duplex that are key to efficient and selective editing.[29] The solution
132 structure of the dsRBDs of ADAR2 bound to the GRIA2 R/G substrate reveals sequence-specific
133 contacts at one of the mismatches and within the hairpin loop.[30] This leaves open the possibility
134 of designing gRNAs that form dsRNA structures that are preferentially bound to ADAR1 and/or
135 ADAR2 dsRBDs.
136
137 Crystal structures of the deaminase domain of ADAR2 have also revealed many characteristics
138 that explain the nuances of ADAR editing. Before the availability of structural data, many deep
139 sequencing studies of A-to-I editing demonstrated that ADARs have certain motif preferences,
140 with the "UAG" sequence motif being favored and a 5' G being disfavored. Like the dsRBDs,
141 the deaminase domain crystal structure detailed dsRNA specific engagement via 5' and 3'
142 binding loops with contacts stretching from 10 bp upstream to 8 bp downstream of the target
143 adenosine.[31, 32] The structure also revealed a disordered 5' binding loop that becomes ordered
144 upon binding to a dsRNA substrate.[31] Interestingly, the ADAR2 5' binding loop is highly
145 conserved across species, yet differs significantly when compared with the ADAR1 5' binding
146 loop which may explain differences in their substrate specificities.[32] Additionally, the crystal
147 structure revealed that ADAR2 acts through a common base flipping mechanism,[33] in which the
148 edited adenosine is flipped out of the duplex and the vacant position is occupied by residue
149 E488, which directly contacts the orphan base. Base flipping allows exposure of the adenosine to
150 the active site to drive deamination.

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151 Initial observations indicated that a hyperactive ADAR2 E488Q mutant gained activity through
152 improved base flipping, not improved binding affinity.[34] The crystal structure revealed hydrogen
153 bonding between E488 and the orphan cytidine, and the pH independence of E488Q may explain
154 the improved base flipping. The crystal structure also provided an explanation for ADAR2
155 disfavoring of a 5' G neighbor, as a 5' G or C could result in a steric clash with ADAR2 G489.[31]
156 Interestingly, a recent crystal structure revealed that a 5' G-G mismatch adopts a non-canonical
157 G_{syn}:G_{anti} hydrogen bonding that alleviates the steric clash and enhances editing of a 5' G
158 adenosine,[35] as had been previously reported.[36] These insights into base flipping and deamination
159 in turn impact gRNA design (which we discuss in more depth in the gRNA design section of this
160 review). More recently, the first crystal structure of the deaminase domain and dsRBD2 engaged
161 to a substrate revealed an asymmetric dimerization via the deaminase domain, and the authors
162 showed many substrates are dimerization dependent.[37] This highlighted a surprising and novel
163 mode of engagement, as previous data provided evidence of dimerization through the dsRBDs.[38]
164 Further work is required to better understand the more complex quaternary structures formed
165 through the deaminase domain and/or the dsRBDs, and how they could inform gRNA design.
166

167 **ADAR Expression and Regulation**

168 ADAR1p110 is ubiquitously expressed. A-to-I editing has been detected at millions of sites
169 within the transcriptome and is present in all tissues and cell types.[39, 40]. A-to-I editing of self-
170 dsRNA mediated by ADAR1 can prevent activation of the cytoplasmic immune sensor, MDA-
171 5.[41] As such, ADAR1 expression is essential for maintaining homeostasis and regulating innate
172 immunity, as evidenced by the severe phenotype of patients with partial loss of function ADAR1
173 mutations leading to Aicardi Goutières syndrome (AGS).[42, 43] Full loss of function mutations to

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174 the deaminase domain have not been identified yet in humans, suggesting that such mutations
175 would be lethal. Indeed, ADAR1 knockout in mice is embryonic lethal.[41, 44] Conversely, ADAR1
176 overexpression is associated with certain cancers,[45, 46] highlighting a potential risk of introducing
177 exogenous ADAR to promote therapeutic RNA editing.
178
179 ADAR1p150 expression is transcriptionally controlled by an interferon responsive element in the
180 promoter region[47] and possesses an N-terminal Z-DNA binding domain as well as an NES. As
181 such, interferon stimulation induces ADAR1p150 expression and localization to the cytoplasm,
182 where it can edit cytosolic dsRNA substrates and alter the RNA editome,[48] playing a key role in
183 viral immunity.[49] Interferon stimulation *in vitro* has been used to improve RNA editing
184 efficiency[6] and the possibility of transient ADAR1p150 induction *in vivo,* for instance, due to
185 innate immune responses to viral infection or drug delivery systems, should be considered when
186 evaluating the specificity of therapeutic RNA editing.
187
188 ADAR2 protein and enzymatic activity are limited to select tissues, such as the brain and
189 heart[50, 51]. ADAR2 plays a key role in site-specific editing for the recoding of amino acids.
190 Murine ADAR2 knockout leads to death several weeks after birth,[52] while the lethal
191 phenotype is rescued by encoding a key RNA editing site within the GRIA2 gene at the
192 genomic level, highlighting the importance of ADAR2 for site-specific editing.
193
194 In contrast to ADAR1 and 2, ADAR3 is exclusively expressed in the brain and lacks deaminase
195 activity.[23, 53] ADAR3 expression negatively correlates with editing and is believed to repress A-
196 to-I editing by competitive antagonism of ADAR1 and ADAR2. This mechanism has been

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197 further characterized in glioblastomas, where ADAR3 competes against ADAR2 for the binding
198 of *GRIA2* transcripts and negatively modulates its editing.[24] ADAR3 knockout mice displayed
199 impaired learning and memory; however, RNA editing at most sites within the transcriptome was
200 unaffected by ADAR3 knockout, with only ten sites showing a statistical difference from wild
201 type, suggesting the regulation of editing may be substrate specific.[54]
202
203 ADAR subcellular localization and transport influence enzyme accessibility to dsRNA substrates
204 and subsequent A-to-I editing. ADARp110- and ADAR2-mediated RNA editing happen co-
205 transcriptionally, and enzyme localization is reported in the nucleus and nucleolus.[55, 56]
206 Alternatively, ADARp150 localizes to the cytosol upon interferon stimulation, where it can
207 access and edit cytosolic dsRNA substrates.[57] It should be noted that this distinction of
208 ADARp110 as a nuclear protein and ADARp150 as a cytosolic protein is an oversimplification
209 as both isoforms are known to shuttle between nucleus and cytoplasm. Nuclear import is
210 mediated by transportin-1 (Trn1), which interacts with an atypical NLS sequence found in the
211 third dsRBD of ADAR1 isoforms.[58] The third dsRBD cannot bind dsRNA and Trn1
212 simultaneously, which makes ADAR1 nuclear import dependent on dsRNA cytoplasmic content.
213 On the other hand, nuclear export of ADAR1p110 and p150 are regulated differently, with
214 ADAR1p110 exported by exportin-5 (XPO5), while the p150 isoform is bound by exportin-1
215 (XPO1) on its NES. Overall, dsRNA content and accessibility in the cytoplasm or nuclear
216 compartment play an important role in sub-cellular localization and subsequent A-to-I editing.
217 Unlike cytoplasmic antisense approaches using RNAi- or RNase H-mediated knockdown,
218 gRNAs that rely on ADARp110- or ADAR2-mediated RNA editing must localize to the nucleus.
219

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220 Beyond the ADAR1/2/3 dsRBD proteins, the human genome encodes for more than 1000 RNA
221 binding proteins (RBPs), sixteen of which contain dsRBDs that may directly compete with
222 ADAR binding: ADAD1/2, CDKN2AIP, DGCR8, DHX9, DICER, DROSHA, ILF3, MRLP44,
223 PKR, PRKRA, SON, STAU1/2, STRBP, TARBP2. Not surprisingly, these double stranded
224 RBPs (dsRBPs) are found to be in the same interactome[59, 60] and share roles in various RNA-
225 related biological processes, such as innate immune response, micro-RNA processing, apoptosis,
226 and cell cycle. They can act with ADAR either synergistically or antagonistically depending on
227 the cellular context.[61] Beyond the biological functions, the crosstalk between different dsRBPs
228 and ADAR highlight the importance of RBP landscape in A-to-I editing. The interaction
229 landscape can be modulated by the cellular context, such as viral infections, UV light, cell cycle,
230 and tissue expression. Thus, the expression levels of dsRBPs are a contributing factor to RNA
231 editing,[40] emphasizing the importance of assaying RNA editing within model systems that reflect
232 the dsRBP expression profile of the therapeutically-relevant target cell.
233
234 A comprehensive picture of the A-to-I editing landscape in human tissues was captured by
235 profiling A-to-I editing in over 50 organs from 8,551 samples of the Genotype-Tissue Expression
236 (GTEx) consortium.[40] ADAR1 and ADAR2 are the only A-to-I mRNA editors known in
237 humans, yet their mRNA expression shows only a moderate correlation with A-to-I editing,
238 depending on the tissue (R^2 = 0.2-0.25 across all tissues with a higher correlation in the of 0.55
239 in the brain). This suggests that additional factors regulate editing. A-to-I regulation can arise
240 from various factors such as RNA splicing, RNA expression levels, and the RBP landscape,
241 which can restrict accessibility to the targeted adenosines. Despite similar editing activity in most
242 tissues, outliers were detected including the cerebellum and arteries with the highest editing

243 levels (potentially explained by high co-expression of ADAR1 and ADAR2) and skeletal
244 muscles demonstrating the lowest editing levels and low expression of ADAR1. Additionally,
245 the authors identified a trans-regulatory mechanism in skeletal muscle via aminoacyl tRNA
246 synthetase complex interacting multifunctional protein 2 (AIMP2), which negatively impacts the
247 stability of both ADAR1 and ADAR2 and may further explain the low editing levels detected in
248 skeletal muscle.[40] Additionally, 3,710 tissue-specific edited sites were identified, and it is widely
249 documented that ADAR1 and ADAR2 have overlapping but unique editing profiles,[34, 62]
250 highlighting the need to engineer and screen gRNAs within disease-relevant models to best
251 reflect the *in vivo* editing environment.
252
253 The ubiquitous expression and activity of ADAR in all human tissues opens the door to many
254 therapeutic applications; however, more work is needed to assess the feasibility of endogenous
255 ADAR recruitment in various tissues. Furthermore, the editing environment within human cell
256 lines is often less active than in the corresponding tissues *in vivo*,[63] and certain models may have
257 limited utility for assessing *in vivo* activity of therapeutic gRNAs.
258

259 CONSIDERATIONS FOR gRNA DESIGN AND OPTIMIZATION

260 As detailed in the preceding section, the underlying biology and structure of ADAR are crucial to
261 developing an RNA editing therapeutic. At the cellular level, a detailed understanding of the
262 expressed ADAR isoforms and trans-regulators is needed to select model systems that reflect the
263 *in vivo* editing environment, while structural knowledge can be leveraged to inform the
264 optimization of gRNA efficiency and specificity. The application of this knowledge and how it

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265 can be leveraged to inform gRNA design and engineering will be described in this section and is
266 outlined in **Figure 2**.
267
268 Broadly speaking, two main delivery approaches for ADAR-recruiting gRNAs have been
269 described: ASOs, which include *in vitro* transcribed or chemically synthesized gRNAs that are
270 delivered directly to the cell; or DNA-encoded gRNAs that are delivered with viral or non-viral
271 technologies, where the gRNA is transcribed upon entry of the exogenous DNA template into the
272 nucleus. Each approach has a set of considerations and ultimately, the delivery modality is
273 influenced by the disease, tissue, and cell type of interest. gRNA design parameters are
274 dependent upon the chosen delivery method and will be discussed independently in the following
275 sections. Regardless of the delivery method, engineering gRNA efficiency and specificity is
276 perhaps the most important element of developing an RNA editing therapeutic and is
277 complicated by the promiscuous activity and innate sequence preferences of the ADAR enzyme.
278
279 Delivery of a DNA-encoded gRNA relies on endogenous cellular transcription to produce the
280 functional gRNA molecule. This drives persistent, durable expression of the gRNA in a natural
281 RNA state. Importantly, a gRNA transcribed from a DNA payload is not limited by the same size
282 constraints as a chemically synthesized ASO, enabling a larger design space to create the ideal
283 target-specific ADAR substrate. Additionally, DNA payloads are amenable to the use of
284 promoters, regulatory elements, and RNA structural modifiers that can be used to tune gRNA
285 expression, persistence, and sub-cellular localization. RBP sequence motifs can also be used to
286 recruit and promote protein interactions that enhance RNA editing (see discussion of RBPs
287 above). Since many human diseases affect terminally differentiated cell populations (*e.g.*,

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288 neurons, muscle cells, etc.), delivery of a DNA-encoded gRNA carries the promise of long-term,
289 durable treatment with a single administration of drug. However, like traditional gene therapy,
290 DNA payloads cannot be simply "turned-off" if an adverse event is experienced, highlighting the
291 need for exquisite specificity and robust non-clinical development data. Depending on the exact
292 delivery method (*e.g.*, AAV, non-viral particles, etc.), immunogenicity and triggering of DNA
293 sensing pathways may limit the overall delivery efficiency and safety. These aspects are not
294 unique to RNA editing and must be considered by the entire gene therapy field.
295

296 As an alternative to DNA-encoded gRNAs, ASOs can be used to recruit ADAR for RNA editing.
297 ASOs can be chemically synthesized with chemical modifications or *in vitro* transcribed from a
298 DNA template. In the case of chemical synthesis, ASOs may be limited by size because of
299 synthesis capabilities and there exists a delicate trade-off between chemical toxicity and drug
300 efficacy. However, numerous advancements in ASO chemistry can improve stability, specificity,
301 and efficiency. With direct administration of these molecules, re-dosing is necessary due to their
302 relatively short half-life, but with certain chemical modifications, molecules may persist for
303 weeks to months.[64] In some contexts, this transient aspect may be an added feature – for
304 example, in the transient modification of a pain receptor. Additionally, ASOs follow more
305 traditional drug pharmacokinetic and pharmacodynamic (PK/PD) profiles and dosing can be
306 stopped if an adverse event is observed.
307

308 For both DNA-encoded gRNAs and ASOs, delivery of the drug to the target tissue and cell type
309 remains a key challenge. Thus, regardless of gRNA design, continued innovation of delivery
310 technologies is required to maximize the therapeutic potential of RNA editing.

311

312 **DNA-ENCODED APPROACHES**

313 **Recruitment of Endogenous ADAR**

314 Programmable RNA editing systems typically consist of two components: the ADAR enzyme
315 and a gRNA that hybridizes to a target mRNA of interest, thereby creating the dsRNA ADAR
316 substrate. Initial efforts in the field of RNA editing relied on overexpression of exogenous
317 ADAR or chimeric enzymes composed of the deaminase domain fused to RNA binding proteins
318 with engineered gRNAs to recruit the enzyme to the target.[9, 15, 65-69] Initially, the gRNA designs
319 typically consisted of two domains, an antisense domain, typically 20-40 nucleotides in length
320 bearing a C-mismatch opposite the target adenosine, and a recruitment domain that brought the
321 ADAR enzyme to the mRNA of interest via a protein-RNA interaction. DNA-encoded gRNAs
322 consisted of a variety of recruitment domains, ranging from a portion of the naturally occurring
323 GRIA2 pre-mRNA hairpin or crRNA:tracrRNA to BoxB and MS2 stem loops, and were utilized
324 to recruit either the wild-type ADAR2 or fusions of the catalytic domains of ADAR to Cas13,
325 λN-peptide and MS2 coat proteins, respectively.[9, 65, 67, 68] Proof-of-concept studies demonstrated
326 the use of AAV-delivered adenosine deaminases in mouse models of Duchenne muscular
327 dystrophy, ornithine transcarbamylase deficiency, and Rett syndrome.[9, 70] While ADAR
328 overexpression based approaches demonstrated the therapeutic potential of RNA editing, the
329 promiscuous nature of ADAR led to transcriptome-wide off-target A-to-I editing[9, 15, 71] with
330 potentially toxic effects seen in mice.[9] To overcome this problem, it is important to restrict the
331 catalytic activity of the overexpressed enzyme only to the target mRNA. By splitting the ADAR2
332 deaminase domain into two catalytically inactive fragments that are brought together by a

333 chimeric gRNA at the given target mRNA to transiently form a functional enzyme, we
334 achieved >100-fold more specific RNA editing as compared to full-length deaminase
335 overexpression.[72] This novel strategy resulted in greatly improved transcriptomic specificity, and
336 the split-ADAR2 system was functional with RNA binding proteins of human origin to limit
337 immunogenicity concerns. Further improvements to the enzymatic activity of the split-ADAR2
338 system or additional protein engineering strategies that enhance specificity may improve its
339 therapeutic potential.
340

341 Even with enhanced specificity of engineered exogenous proteins, this approach will still be
342 challenged by packaging limits of the delivery modalities (e.g., AAVs) and immunogenicity
343 concerns. Therefore, recruitment of endogenous ADAR to perform targeted RNA editing is the
344 preferred approach. We recently demonstrated the use of DNA-encoded gRNAs for the
345 recruitment of endogenous ADAR to mediate RNA editing.[9] While gRNAs with antisense
346 domains as short as 20 nucleotides sufficed to recruit overexpressed ADAR, increasing the
347 length, for example, to 60 nucleotides or greater, enabled recruitment of endogenous ADARs.[9]
348 This was an important advancement of the technology as it opened the door to potential
349 therapeutic applications.

350 **gRNA Expression, Stability, and Localization**

351 DNA-encoded gRNAs can be further optimized by focusing on expression, stability, and
352 localization. gRNAs are typically transcribed from pol III promoters (*e.g.*, U6) and lack a 5′ cap
353 and a 3′ poly-A tail, leaving them vulnerable to 5' and 3' exonucleases, thereby reducing their
354 half-lives. Given that RNA editing is a transient event that dilutes out with mRNA turnover, it is
355 important to improve expression and stability of the U6 transcribed gRNA. Circularization of

356 RNA is one strategy to prevent exonuclease digestion and increase RNA half-life. Towards this
357 end, we created DNA-encoded circular gRNAs by flanking long antisense domains with twister
358 ribozymes.[8, 73] Upon transcription, the twister ribozymes self-cleave, leaving specific overhangs
359 that are recognized and ligated by the ubiquitously expressed RtcB RNA ligase to form a circular
360 gRNA.[74] The use of circular gRNA greatly improved the persistence of RNA editing over linear
361 gRNAs both *in vitro* and *in vivo.* While no editing of the PCSK9 3'UTR in mice livers was
362 detectable via AAV-delivered linear gRNA, 11% editing was detectable via AAV-delivered
363 circular gRNA. By packaging two copies of the U6 promoter and circular gRNA within an AAV,
364 RNA editing levels increased to 53% at eight weeks post injection.[74] Additionally, AAV-
365 delivered circular gRNAs were utilized to repair a premature stop codon (W392X) in the alpha-
366 L-iduronidase mRNA in the liver of a mouse model of Hurler syndrome, via recruitment of
367 endogenous ADAR enzymes, resulting in 12% RNA editing and partial restoration of enzyme
368 activity.[8,11] Although short-term studies showed no toxicity in mice with RNA editing levels
369 being maintained up to 8 weeks post injections, longer studies are needed to assess the safety and
370 durability of AAV-delivered circular gRNAs.
371
372 An alternate strategy to improve gRNA stability is the use of natural exonuclease-resistant
373 structures at the 5' and/or 3' ends of the gRNA.[75-77] Advances in the field of siRNA and CRISPR
374 gRNAs have demonstrated the utility of this approach in enhancing the stability of U6
375 transcribed RNA.[78, 79] These learnings from the fields of CRISPR gRNAs and antisense RNAs
376 can be applied to enhance the performance of the ADAR-recruiting gRNAs. Furthermore,
377 focused efforts need to be made to engineer spatio-temporal regulation of RNA editing. The use
378 of tissue-specific enhancer elements will allow for modulation of RNA editing activity in space

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379 while engineering small molecule-based regulation of gRNA activity could enable temporal
380 control.[80, 81]

381 **gRNA Structure and Interaction with mRNA**

382 While gRNA abundance is an important factor contributing to the efficiency of RNA editing,
383 intrinsic characteristics of the gRNA, such as intramolecular secondary structure and nucleotide
384 composition, also play a major role in influencing the activity of a gRNA. Most transcribed
385 gRNAs are relatively long (greater than 40 bp) and can have complex secondary structures. The
386 secondary structure of a gRNA affects its ability to bind its target and the use of computational
387 tools to predict intramolecular secondary structure can improve gRNA designs. Additionally, the
388 editing of adenosines on the gRNA itself could impact editing of the target adenosine. RNA
389 editing via ADARs can occur on both strands of an RNA duplex, thereby altering the sequence
390 of the gRNA itself. This could, in turn, impact the ability to effect ADAR-mediated editing of
391 additional target transcripts.[82] Conversely, the secondary structure of the target pre-mRNA and
392 position of the editing site within the transcript, such as the untranslated (UTR) versus coding
393 (CDS) regions, may also impact editing. As observed in the ASO and RNAi fields, many regions
394 within an mRNA are amenable or refractory to knockdown due to accessibility. However, these
395 knockdown strategies have the luxury of tiling across the mRNA to identify the optimal location
396 for knockdown. RNA editing may prove challenging if a target adenosine lies within a highly
397 structured or inaccessible region of an mRNA, making it difficult to edit. It remains to be
398 determined whether longer gRNAs or gRNAs that employ two or more discontinuous
399 hybridization regions could modulate the target RNA structure to help access adenosines located
400 in such regions.[7] A more systematic approach comparing the accessibility of ASOs and gRNA-
401 mediated RNA editing would help to better understand the limitations imposed by the target

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402 mRNA structure. Further, the entire dsRNA stretch formed between the gRNA and target mRNA
403 becomes a substrate for the ADAR enzyme. Thus, further engineering of the gRNA is essential
404 to achieve specific editing of the target adenosine.

405 **Engineering Specificity**

406 The ability to recruit endogenous ADAR limits the issue of transcriptome-wide off-target
407 editing; however, bystander editing of non-target adenosines within the gRNA-target complex is
408 commonly observed. As discussed, ADAR enzymes have promiscuous editing activity as
409 evidenced by their role in regulating innate immune responses to dsRNAs and the millions of
410 identified editing sites within the transcriptome.[51, 83, 84] Despite the promiscuous nature of
411 ADAR, many natural substrates have been identified that are edited with high selectivity and
412 efficiency for the purpose of modulating protein function by recoding at the amino acid level or
413 altering pre-mRNA splicing.[85-87] It is hypothesized that secondary structural features within the
414 dsRNA can drive efficient and selective editing of these substrates. Secondary structural features
415 downstream of the edited adenosine within the GRIA2 R/G substrate have been shown to
416 increase editing efficiency,[29] while the addition of secondary structures was shown to limit the
417 promiscuous nature of ADAR activity within a dsRNA substrate.[88] Mutagenesis and high-
418 throughput screening of natural substrates within NEIL1, TTYH2, and AJUBA pre-mRNA have
419 demonstrated the impact that secondary structure can have on editing efficiency.[89] In addition,
420 high-throughput screening of secondary structures within long dsRNA substrates mapped ADAR
421 activity 30 nucleotides upstream of secondary structure disruptions and displayed a periodicity to
422 editing.[82] Furthermore, co-immunoprecipitation and RNAseq showed a periodicity to ADAR
423 engagement to natural substrates occurring in 50 nucleotide increments.[90] These observations
424 may be leveraged to engineer gRNAs with improved specificity required for therapeutic

425 applications. However, these features are observed within a cis RNA interaction, and it remains
426 to be seen how easily they will port into the trans interaction of a gRNA and target RNA.
427
428 We recently used secondary structural features to address the issue of bystander editing. We first
429 demonstrated that a perfect complementary gRNA containing a C mismatch across the target
430 adenosine mediates numerous bystander editing events driven by endogenous ADAR. Others
431 have shown that incorporation of a G mismatch positioned at bystander adenosines can reduce
432 off-target ADAR activity, but the RNA editing efficiency of the target adenosine may be
433 negatively impacted.[10] As an alternative approach, we incorporated internal loops in specific
434 positions along the entire length of the gRNA. This eliminated promiscuous RNA editing
435 without affecting the efficiency of the target adenosine,[8] and a similar approach using
436 discontinuous stretches of hybridization also improved specificity.[7] Another approach
437 demonstrated that precise nucleotide deletions across bystander adenosines can lead to improved
438 specificity of circular and linear gRNAs.[11] We anticipate that additional refinements to gRNA
439 design will further reduce bystander editing and boost target editing efficiency.

440 **Delivery of DNA-Encoded gRNAs**

441 Currently, there is a limited clinically validated toolset for the delivery of DNA payloads; thus,
442 despite challenges, the gene therapy field relies heavily on adenoviruses and AAVs. Preclinical
443 *in vivo* proof-of-concept studies for ADAR-based RNA editing have used AAVs to deliver
444 DNA-encoded gRNAs to mice livers. The natural tropism of many AAV serotypes lends itself to
445 targeting disorders of the liver, muscle, CNS, and eye. However, ADARs are ubiquitously
446 expressed ,and ongoing efforts to expand the tropism and specificity of AAV serotypes is an
447 active area of research that might enable the delivery of gRNA to additional tissue types[91-96]. For

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448 example, efficient delivery of AAV to the CNS requires invasive techniques such as direct
449 injection into the brain parenchyma. Delivery vectors with the ability to efficiently cross the
450 blood brain barrier and transduce the CNS would increase safety and simplify the design and
451 execution of preclinical and clinical studies. However, systemic injection of AAV results in high
452 transduction of the liver. Thus, reducing liver uptake while increasing transduction of the target
453 organ may improve safety and efficacy. In addition to viral delivery, non-viral approaches, such
454 as lipid nanoparticles (LNPs), can be used to deliver DNA payloads but, as with AAV, primary
455 uptake is in the liver. As delivery technologies improve, new therapeutic opportunities will
456 emerge for RNA editing.

457 **ANTISENSE OLIGONUCLEOTIDES FOR RNA EDITING**

458 ASOs are another widely used approach for therapeutic RNA editing that builds on decades of
459 work in the oligonucleotide chemistry field. ASOs have progressively undergone three major
460 improvements: the introduction of phosphorothioate backbone chemistry, the use of sugar
461 modifications such as 2'-O-methyl, and the use of nucleic acid analogs, such as locked nucleic
462 acids (LNA).[97] In combination, these improvements have enhanced stability, efficiency,
463 biodistribution, cell penetrance, and safety resulting in enormous growth in oligonucleotide-
464 based therapeutics in the last two decades. Currently, there are greater than fifteen ASO-based
465 therapies that have reached late-stage clinical testing or received FDA approval.[98, 99] Importantly,
466 lessons learned from the ASO field can be leveraged for the design and clinical application of
467 chemically-synthesized gRNAs for therapeutic RNA editing.
468

469 Many of the challenges shared broadly by the ASO field, including delivery, biodistribution, cell
470 penetrance, and safety, are similarly applicable to RNA editing. Additionally, a few challenges

471 unique to RNA editing exist and include gRNA length, potentially distinct interactions of the
472 ADAR enzyme with ASO chemistry, and nuclear delivery and localization. In the contexts of
473 RNase H-mediated degradation or exon skipping and siRNAs for RNAi-mediated knockdown,
474 short oligos of ~ 20 nucleotides are effective. However, ASOs to mediate RNA editing will
475 likely require at least 30 nucleotides[12, 13] and the use of recruitment domains could further
476 increase the length to 60 - 90 nucleotides.[6] In addition to length, ideal gRNA structures that
477 balance stability while promoting ADAR binding and enzymatic activity will be key to
478 maximize RNA editing efficiency and specificity. Similarly, ASO stability was optimized with
479 "gapmers" that modified structural features while still retaining RNase H-directed activity.[100]
480 Lastly, while RNase-H activity can occur in the nucleus or cytoplasm,[101] and RNAi-mediated
481 knockdown occurs in the cytoplasm,[102] most RNA editing occurs co-transcriptionally in the
482 nucleus.[103] Thus, nuclear delivery and localization of the chemically-modified gRNA are
483 important parameters to achieve efficient RNA editing.

484 **Key Advancements for ASOs and RNA Editing**

485 Many key ASO advancements have been adopted by the field of RNA editing and numerous labs
486 have used ASOs to recruit endogenous ADAR to edit target adenosines *in vitro* and *in vivo*.[104-106]
487 An early application was demonstrated using an exogenous ADAR deaminase domain covalently
488 linked to an ASO that directed the deaminase domain to a target mRNA.[66] Building on this early
489 work, recruitment of endogenous ADAR was achieved using chemically-modified ASOs with an
490 antisense domain attached to a portion of the GRIA2 R/G hairpin.[6] This method demonstrated
491 RNA editing across multiple mRNA targets in cell lines and primary cells. Chemical
492 modifications included 2'OMe groups throughout much of the ASO, aside from the three-
493 nucleotide motif across from the target and select locations within the GRIA2 R/G hairpin;

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494 phosphorothioates at the 5' and 3' ends, and LNAs at the 3' end. The use of chemical
495 modifications was crucial for the recruitment of endogenous ADAR, as an unmodified ASO
496 resulted in no detectable editing unless the cells were treated with IFN-alpha to induce ADAR
497 p150 expression. Editing of two therapeutically relevant targets was also demonstrated:
498 introduction of T701C in STAT1 to prevent phosphorylation and downstream signaling of thee
499 JAK-STAT pathway,[107] and correction of the PiZZ mutation (E342K) in SERPINA1, the most
500 common cause of α1-antitrypsin deficiency (AATD).[108] 21% editing of STAT1 and 10-18%
501 editing of the E342K codon within a SERPINA1 cDNA minigene was achieved using
502 chemically-modified ASOs. Interestingly, the optimal ASO design was 91 nucleotides in length,
503 which included a 38-nucleotide antisense domain and 53-nucleotide hairpin structure. This is far
504 longer than the ~20 nucleotide ASOs used for RNase H-mediated knockdown and exon skipping,
505 and longer designs may complicate delivery, manufacturing, and safety. It is also important to
506 note that bystander editing was observed at the neighboring 3' adenosine of the SERPINA1
507 target. The introduction of a 2'OMe group on the paired uracil within the ASO was able to
508 reduce this bystander editing, albeit with a concurrent reduction of editing at the target
509 adenosine. A similar tradeoff was observed with the use of A-G mismatches to reduce bystander
510 editing for DNA-encoded gRNAs.[10] Further work is needed to understand the basic design
511 principles to optimize both efficiency and selectivity of editing. An alternative strategy used a
512 much longer ASO of 100 nucleotides and utilized 2'OMe modifications on the 5' and 3' ends.
513 Using this design to target the PPIB transcript yielded 20% editing in human T cells, while the
514 unmodified ASO failed to produce detectable editing.[10] These studies clearly demonstrated the
515 potential of ASOs in eliciting efficient and specific RNA editing using the endogenous human
516 ADAR enzyme.

517

518 Given the known structural details of the ADAR footprint and the size of many natural substrates
519 that result in recoding of amino acids, which often contain less than 30 base pairs of dsRNA, it is
520 not surprising that recent publications have significantly shortened the length of ASOs. One
521 recent publication demonstrated the use of a 30 nucleotide stereopure ASO with
522 phosphorothioate backbone.[12] The footprint of the design matches the canonical asymmetric
523 footprint of ADAR, with approximately 5 base pairs on the 5' side of the target to accommodate
524 the 5' binding loop of the deaminase domain, and approximately 25 base pairs on the 3' side of
525 the target to accommodate the 3' binding loop of the deaminase domain, along with the dsRBDs.
526 Additionally, their ASO design contained extensive use of 2'-fluoro modifications on the 5' end,
527 2'-O-methyl on the 3' end, and deoxyribonucleotides across from the edit site, indicating ADAR
528 is tolerate of these modifications in their respective locations. The stereopure ASOs achieved
529 robust editing in tissue culture and *in vivo*. A liver-targeting GalNac-ASO conjugate was
530 intravenously administered to non-human primates (NHP) and achieved up to 50% editing for a
531 non-clinical target in the 3' UTR of the endogenous ACTB transcript. While the target adenosine
532 lies in an ADAR-favored UAG motif, this data in NHPs supports the translatability of RNA
533 editing. A single dose showed persistent RNA editing 50 days post-injection, further highlighting
534 the therapeutic potential of GalNac-ASO conjugates. In the context of a disease-relevant target,
535 stereopure ASOs achieved ~75% editing of the SERPINA1 E342K mutation *in vitro*. Shortening
536 the gRNA to 30 nucleotides simplified the manufacturing, and the lack of a hairpin recruitment
537 structure means the ASO engagement with ADAR is dependent on target hybridization and is
538 less likely to perturb natural ADAR function. Furthermore, shorter length reduces the risk of
539 chemical toxicity that appears to be a class effect with high-dose, chemically modified ASOs.[109]

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540

541 Knowledge of ADAR structure and function can also be leveraged to better inform ASO design.
542 A clever "bump-hole" design paired an engineered ADAR2 E488Y mutant with an ASO
543 containing an abasic site across from the target adenosine.[69] Due to a steric clash, the ADAR2
544 E488Y mutant had low enzymatic activity; however, the abasic site resolved this clash and
545 restricted its activity to the ASO-target complex formed upon hybridization to the target mRNA.
546 This strategy could enable the use of exogenous ADAR while minimizing off-target editing but
547 comes with the complication of delivering a non-human protein with the risk of an antidrug
548 response to the ADAR2 E488Y. More recently, the same group detailed the rational design of
549 ASOs for the recruitment of endogenous ADAR.[13] The ADAR2 E488Q mutation has been well
550 documented to improve editing through hydrogen bonding of Q488 to the orphan base in a pH-
551 independent manner.[31, 110] Inspired by this observation, the researchers sought to improve
552 hydrogen bonding from the orphan base on the ASO with the wild-type ADAR2 E488. Indeed,
553 incorporation of the cytidine analog 2'-deoxy Benner's base Z (dZ), which was hypothesized to
554 have a favorable hydrogen bond pattern with E488, improved the biochemical reaction rate
555 kinetics of both wild-type ADAR1 and ADAR2 three-fold. When tested in human ARPE-19
556 cells, incorporation of dZ at the orphan position of the ASO improved editing of a γ-secretase
557 cleavage site within the APP transcript from 6% to 19%. There is still much to be learned about
558 the principles behind chemical modifications and how they impact ADAR substrate engagement
559 and deamination, but these results highlight the potential of rational ASO design to augment the
560 interaction and enzyme kinetics of endogenous ADAR.

561

562 These data indicate that ASOs are a viable and promising path for therapeutic RNA editing. In
563 the short-term, ASO delivery to the liver, muscle, kidney, or direct injection to the CNS are
564 viable options. Unlike the long-term persistence of DNA-encoded gRNAs resulting from AAV
565 delivery, ASO half-life allows for transient editing and redosing as needed, and the dose can be
566 optimized to fine-tune the desired amount of editing required for the therapeutic effect. Ongoing
567 work within the ASO field to address the challenges associated with delivery, biodistribution,
568 and cell penetrance will quickly be adopted and applied to RNA editing. Meanwhile, additional
569 work to optimize and standardize ASO designs for the recruitment of endogenous ADAR is
570 needed.

571

572 **THERAPEUTIC OPPORTUNITIES**

573 RNA editing provides many attractive therapeutic applications, the most logical being correction
574 of G-to-A missense and nonsense mutations, of which ~7,000 pathogenic G-to-A mutations are
575 reported in ClinVar (accessed on April 13, 2022). In support of therapeutic RNA editing, several
576 proof-of-concept *in vivo* studies using ADAR-mediated RNA editing to correct missense and
577 nonsense mutations have been described. In a mouse model of Hurler syndrome, endogenous
578 ADAR was recruited to correct a nonsense mutation in the IDUA transcript and restore protein
579 function.[8,11] In two mouse models of Rett syndrome, RNA editing using exogenous ADAR was
580 able to correct both nonsense (MECP2^{W104X}) and missense (MECP2^{R106Q}) mutations.[20, 70]
581 Correction of a nonsense mutation in the *mdx* mouse model of Duchenne muscular dystrophy
582 was achieved with exogenous ADAR recruitment.[9] Additionally, RNA editing of a 5' splice site
583 missense mutation in the spf^{ash} mouse model of ornithine transcarbamylase deficiency restored
584 correct splicing *in vivo,*[9] highlighting an ability of the technology to function at the pre-mRNA

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585 level. Lastly, significant attention has been directed on the SERPINA1 E342K mutation that
586 causes AATD and two independent groups have demonstrated >40% RNA editing of mutant
587 SERPINA1 within human cells using ASOs.[6, 12]
588

589 Beyond correction of point mutations, targeting adenosine-containing motifs such as splice
590 acceptor sites, translation initiation sites, polyadenylation signals, and microRNA binding sites
591 can modulate mRNA and/or protein levels for therapeutic purposes. ADAR plays a natural role
592 in the regulation of splicing[111] and genomic editing of splice sites is able to modulate splicing,[112-
593 114] strengthening the rationale for therapeutic splice site targeting. Furthermore, ASOs and DNA-
594 encoded antisense RNAs have been used to mask and block the function of splice sites,[115]
595 polyadenylation sites,[116-118] TISs,[119] upstream open reading frames (uORFs),[120] and microRNA
596 binding sites.[121] Therefore, gRNAs designed to both mask and edit these regions may provide an
597 additive effect. Indeed, many of these motifs have been hardwired at the genomic level by DNA
598 editing[122-124] and have conferred the desired molecular effect.
599

600 Further applications for RNA editing can also be envisioned. The advent of monoclonal
601 antibodies[125] created a new instrument to block protein function and signaling by binding to
602 soluble proteins and membrane proteins, such as TNF-alpha[126] and HER2[127], respectively.
603 However, intracellular proteins are inaccessible to antibodies, and complex membrane proteins
604 pose a challenge to antibody discovery, such as ion channels and GPCRs. ADAR-mediated RNA
605 editing can introduce 17 different amino acid substitutions that can be used to modulate protein
606 function and abolish or enhance protein-protein interactions. This may be of particular interest
607 for proteins that are not amenable to antibody therapy. For example, RNA editing of the BACE

608 cleavage site on APP was demonstrated in ARPE-19 cells,[13] a potential target for the treatment
609 of Alzheimer's disease.[128] Additionally, endogenous ADAR2 plays a central role in modulating
610 ion channel permeability,[129] and extending this function to therapeutic regulation of ion channels,
611 such as Nav1.7, is of great interest.[130, 131]

612

613 The therapeutic targets mentioned above could also be corrected at the genomic level using DNA
614 editing technologies; therefore, one must consider the risk/benefit profile of DNA vs. RNA
615 editing when selecting a therapeutic approach for any given disease indication. First and
616 foremost, DNA-modifying enzymes create permanent changes that impact 100% of transcribed
617 RNAs at both on- and off-target sites. In contrast, RNA editing is transient in nature for the life
618 of the edited RNA molecule and can be tuned to the desired fraction of RNA molecules to be
619 edited within a cell. For therapeutic applications that require a transient pharmacodynamic effect,
620 such as the treatment of acute pain, obesity, viral infection, and inflammation, it would be
621 undesirable to introduce permanent alterations to the genome. Thus, the transient modulation of
622 protein expression or function by RNA editing is advantageous. Additionally, the tunability of
623 RNA editing can be exploited where partial knockdown or partial protein modulation is desired.
624 In fact, many endogenous ADAR dsRNA substrates that are edited for the purpose of recoding
625 show a significant range in editing efficiency, from single digit to 100%.[18] Some organisms have
626 even evolved techniques to fine-tune RNA editing based on their environment.[132-134]
627 Mutagenesis studies have demonstrated that altering the secondary structure of natural substrates
628 can increase or decrease editing,[89] highlighting once again the importance of gRNA design for
629 therapeutic application of RNA editing.

630

631 RNA editing offers unique safety and delivery advantages over DNA editing. Despite the
632 potential and early clinical success of CRISPR/Cas DNA editing technologies,[135] safety concerns
633 persist.[136] RNA editing does not cause permanent alterations at the genomic level, avoiding the
634 oncogenic risk associated with DNA editing and as discussed above, allowing for transient
635 treatment of acute conditions. Additionally, a single gRNA payload is sufficient to recruit
636 endogenous ADAR. This is in contrast to DNA editing systems that rely on the use bacterial
637 proteins or hyperactive enzymes that carry the risk of immunogenicity[137, 138] and present delivery
638 challenges due to their size. Because ADAR is ubiquitously expressed, its potential within any
639 organ or cell type is only limited by the delivery of the gRNA to the target cell. This includes
640 non-dividing cells, such as neurons in the CNS, where the lack of homology-directed repair
641 (HDR) pathways limit the use of certain DNA editing technologies. ADAR-mediated RNA
642 editing is limited to A-to-G changes, and efforts to utilize APOBEC1 for C-to-U RNA editing
643 are less advanced.[139] In contrast, improved DNA editing technologies, such as base editing and
644 prime editing, can introduce mutations not feasible with current RNA editing technologies, and
645 circumvent the need for HDR required by traditional CRISPR/Cas9 methods. The ability for
646 permanent genomic alterations also makes DNA editing particularly attractive in rapidly dividing
647 cells or progenitor cells and has been extensively used in *ex vivo* cell therapy applications.
648 Overall, given the many differentiators highlighted above, RNA editing has great potential as a
649 therapeutic modality across a wide range of challenging diseases and has become an important
650 part of the biotechnology molecular toolkit.
651
652 The field of RNA editing will continue to gain traction from advances in delivery technology as
653 new AAV capsids and ASO modifications expand the tropism and penetrance of different

654 tissues. Meanwhile, advances to gRNA discovery and design may open new opportunities.
655 Increased knowledge of ADAR and gRNA structure will allow for more sophisticated design,
656 such as the recoding of multiple codons within a transcript, as seen for natural substrates.[87] The
657 limited cargo size of a gRNA expression cassette could easily allow for multiplexing and editing
658 of multiple transcripts. One could envision targeting multiple pathways or engineering both
659 interfaces of a protein-protein interaction. The potential for new modalities has also emerged.
660 RNA editing is being leveraged for RNA sensing, allowing expression of a payload to be gated
661 on the transcriptional stage of the target cell.[140-142] However, to make any of these possibilities a
662 reality, early proof-of-concept studies may need to be improved to translate the results into the
663 clinic.

664 **TRANSLATION TO CLINIC**

665 Increased understanding of the fundamental biology and control of RNA editing has advanced
666 this technology to the cusp of clinical application. Successful translation to the clinic requires
667 addressing remaining challenges. Several regulatory guidance documents are available that
668 broadly address many of the challenges facing sponsors during development of gene therapies
669 and regenerative medicines[143-153] These guidance documents encompass novel platform
670 technologies such as RNA editing and represent current regulatory thinking on research
671 pharmacology, nonclinical safety, product manufacture/characterization, and clinical assessment.
672 While these guidance documents can generally be applied to RNA editing, there remain
673 technology-specific issues requiring careful consideration during development.
674

675 RNA editing must be exquisitely selective for the intended RNA target, with biologically
676 negligible off-target editing. This is required to achieve the intended pharmacological activity

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677 and minimize safety risks. Validated methods for screening on- and off-target editing are
678 required to determine the specificity profile of any given gRNA. Deep RNA sequencing can
679 characterize global alterations in the cellular transcriptome and establish the editing signature of
680 a gRNA.[51, 154] Establishing this signature across the relevant cell and tissue types, influenced by
681 the expected biodistribution of a given delivery modality, will be critical to predicting clinical
682 safety risk. Previous murine studies that introduced exogenous ADAR or hyperactive forms of
683 ADAR showed a significant increase in off-target editing,[9, 15, 20, 70] while recent publications that
684 redirect endogenous ADAR to the target of interest have minimal off-target editing.[8, 11, 12] Should
685 specific transcripts demonstrate elevated levels of off-target editing, further characterization to
686 understand any physiological or toxicological consequences may be required. In some instances,
687 off-target editing may not affect protein-level expression or function, such as in the case of an
688 edit leading to a synonymous codon change that does not alter the structure of the translated
689 protein. In other cases, off-target edits could be significantly disruptive, for example in the
690 introduction of a non-synonymous mutation leading to a gain or loss of function to the protein.
691 Given the spectrum of outcomes from potential off-target editing, it is important to consider the
692 impact of those edits on a case-by-case basis, particularly since different pathways will have
693 varying tolerance for perturbation. At a minimum, the relationship between off-target editing and
694 protein expression should be established and followed up by functional studies to investigate the
695 impact to known downstream pathways. The broader consequences of off-target editing at the
696 tissue and organism levels will be evaluated in the toxicology studies required by regulatory
697 agencies, but analyses in relevant human cells may aid in interpretation of findings.
698

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699 Similar to strategies used to assess DNA editing off-target events,[155] deep sequencing methods
700 can also be used to determine whether there have been changes to the endogenous editome of the
701 cell as a result of preferential ADAR recruitment to gRNA-targeted sequences.[81] Long-term
702 disruption of natural ADAR function could have immunological consequences and impact a
703 number of cellular pathways. It should be noted that the transcriptome has millions of A-to-I
704 editing sites[39] spread across thousands of dsRNA substrates.[156] It is unlikely that the addition of a
705 single substrate would perturb ADAR activity; however, gRNA designs that include a
706 recruitment domain[6, 7] are capable of binding ADAR independent of hybridization to the target.
707 This poses a greater risk of perturbing ADAR activity, especially if expressed at high levels.
708

709 Additionally, when assessing the potential for off-target effects it is important to consider the
710 relative contributions of ADAR1 and ADAR2 towards therapeutic editing. Each enzyme is
711 capable of efficient and selective editing of natural substrates for recoding at the amino acid
712 level, yet subtle differences in their preferential editing based on sequence context and secondary
713 structure exist. [26, 62] For example, therapeutic editing in the liver would primarily rely on
714 ADAR1, while biodistribution to tissues with high ADAR2 expression (*e.g.*, brain) [40] may result
715 in altered editing efficiency or specificity of the target mRNA. Ensuring the gRNA is selective
716 for the target adenosine in both an ADAR1 and ADAR2 environment is an important
717 consideration, especially when the delivery modalities may lack specificity for the target
718 tissue(s). Engineered cell lines that express ADAR1 and ADAR2 in isolation can be a valuable
719 tool to assess the relative specificity of each enzyme for gRNA mediated editing of the target
720 mRNA.[7]
721

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722 Much attention is focused on quantifying RNA editing at the transcript level, but equally crucial
723 is ensuring this leads to a corresponding change in protein that imparts the desired phenotypic
724 outcome. It is often assumed that correction of a missense mutation will lead to a corresponding
725 level of corrected protein; however, this may not always be the case. Although inosine is
726 interpreted as guanosine, there is a small loss in fidelity that can vary based on sequence context,
727 and the presence of more than one inosine can stall translation.[157] Therefore, it is desirable to
728 quantify both RNA editing and protein restoration in preclinical safety and efficacy assessments.
729

730 gRNA delivery, whether through an ASO or DNA-encoded approach, is an important factor in
731 maximizing exposure and activity in the cells of interest and minimizing off-target exposure and
732 expression that could contribute to unwanted side effects. An optimal delivery approach should
733 enable efficient tropism, cellular uptake, and cell type-specific expression and function. The
734 method of delivery will impact nonclinical, manufacturing, clinical, and regulatory
735 considerations for RNA editing drug development. The gRNA itself is sufficiently compact to be
736 developed as a chemically-modified oligonucleotide, analogous in many ways to several
737 commercially-approved ASO examples.[158] This approach would likely involve repeated dosing
738 to achieve a persistent effect, and may be restricted in its therapeutic application based on the
739 natural pharmacokinetic and biodistribution properties of the ASO. Alternatively, viral vectors
740 such as AAV can be used to deliver DNA-encoded gRNAs; this offers the potential for persistent
741 gRNA expression with just a single dose. AAV vectors have shown promise for durable gene
742 expression across a range of indications in the clinic, with approved products in the United States
743 for inherited retinal dystrophy in 2017 and spinal muscular atrophy in 2019.[159] AAV capsids can
744 be engineered to drive tissue-specific tropism that would enable vectorized delivery of gRNAs

745 with targeted biodistribution.[160] Translation of AAV-based drug products comes with well-
746 known challenges in manufacturing and safety that must be taken into consideration during
747 development.[161, 162] In particular, the immune response to AAV vectors generally precludes
748 repeated administration in the same patient, and some high-dose clinical trials have led to severe
749 adverse events. However, there are several strategies currently being explored to circumvent or
750 lessen the impact of this immune response, including immunosuppression regimens, use of
751 immune orthogonal AAVs, and capsid engineering to enable lower doses.[163, 164] Achieving
752 maximum payload delivery to the target cells while minimizing exposure of non-target cells can
753 reduce drug manufacturing costs and patient dosing requirements, which could translate into
754 reduced toxicity risks.
755
756 Regardless of the delivery method selected, a comprehensive characterization of vector and
757 gRNA tissue biodistribution and expression profile in relevant nonclinical models is expected to
758 enable first-in-human dosing. Because the transcriptome differs across tissues, the biodistribution
759 data can highlight cells and tissues of particular interest when assessing efficacy and tolerability.
760 Biodistribution/expression data can be used in conjunction with on- and off-target editing data in
761 relevant model systems to project dosing requirements needed to achieve a therapeutic benefit
762 and a safety margin to derive an initial clinical dose and dose escalation strategy. In some cases,
763 this data may need to be extrapolated across multiple model systems. For example, healthy large
764 animal models typically used for nonclinical toxicology studies may not have the desired target
765 mutations that enable a readout of on-target RNA editing efficiency, but they can inform on dose
766 response and gRNA biodistribution. A dose response for editing could then be extrapolated from
767 a disease model that has a relevant on-target mutation, using biodistribution/expression data in

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768 the target tissue to connect the model system readouts. Therapeutic dosing strategies will thus be
769 highly dependent on indication and model systems available and will be a critical topic for
770 discussion with regulatory agencies during preclinical development.
771
772 **Clinical Development**
773 From a clinical perspective, each disease indication, target organ, and delivery modality will
774 influence the clinical development plan and ultimately the information that can be learned from
775 early-phase clinical trials. There is a desire to quantify both RNA editing and protein influence
776 from tissue biopsies to inform dose selection; however, the complexity of clinical biopsies differs
777 across tissues. For instance, biopsies of the liver come with risks and are less frequently done.
778 Similarly, biopsies from the CNS are generally not feasible. In the case of muscle, biopsies are
779 more routinely performed and may enable a comparative analysis of RNA editing, protein
780 correction or restoration, and phenotypic change in the clinical study. This information will be
781 key in dose escalation studies to identify the minimal dose required for a therapeutic impact. In
782 tissues where biopsies are not feasible, understanding the relationship between RNA editing,
783 protein modulation, and phenotypic outcome must be clearly established in preclinical studies,
784 and careful consideration is required when selecting the appropriate dose and readouts for human
785 studies.
786
787 Correction of missense and nonsense mutations is the most logical application for RNA editing.
788 Numerous groups have demonstrated preclinical data targeting a missense mutation in
789 SERPINA1 leading to AATD[6, 12] (as reviewed above). The G-to-A SNV encoding the E342K
790 mutation affects > 100,000 people worldwide,[108] creating a large unmet medical need. The

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791 mutation causes a toxic gain of function, and aggregated protein accumulates in hepatocytes.
792 Additionally, reduced AAT secretion from hepatocytes into the serum causes neutrophil elastase,
793 the AAT natural substrate, to accumulate in the lungs.[108] Due to this combined gain and loss of
794 function in the liver and lungs, attempts at knockdown, gene replacement, or protein therapy
795 have fallen short, since they do not address both aspects of the disease. AATD is well suited for
796 therapeutic RNA editing. The liver is an ideal target organ for delivery of DNA or RNA
797 payloads, and correction of the SNV at the RNA level can retain endogenous expression levels
798 while reducing toxicity in the liver and increasing secretion to the serum. Lastly, a clear
799 benchmark of >11 µM AAT in the serum has been established to restore its function in the
800 lungs. This provides a great opportunity to establish RNA editing as a new therapeutic modality
801 and address a large, unmet medical need.
802
803 As RNA editing becomes established in the clinic, we anticipate refined use to treat indications
804 with smaller patient cohorts, eventually enabling truly personalized medicine, similar to recent
805 examples with ASOs. In one case study, deep sequencing of a pediatric patient suffering from
806 Batten's Disease revealed a pathogenic splice variant in the MFSD8 gene leading to a premature
807 termination codon. An ASO was quickly designed to mask the cryptic splice acceptor site and
808 restore the use of the canonical splice acceptor site. Within one year of diagnosis, the drug was
809 designed, manufactured, and administered to a single patient, who displayed reduced symptoms
810 after treatment.[165] Current delivery technologies for DNA payloads are not yet amenable to
811 individualized treatment, but the ease of ASO synthesis may facilitate the small-scale
812 manufacturing needed for wider adoption of personalized medicine. As knowledge of gRNA

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813 design principles improves, we anticipate similar scenarios unfolding for patients with rare,
814 pathogenic G-to-A SNVs.
815
816 **CONCLUSION**

817 RNA therapeutics based on ASOs and RNAi that enable programmable RNA knockdown are
818 already having considerable impact on human medicine. The recent advent of ADAR-based
819 technologies that add programmable RNA editing to the molecular toolkit has created new
820 possibilities in transcriptome engineering. By enabling direct nucleotide-level modulation of
821 endogenous RNA transcripts and correspondingly, an ability to modulate RNA substrates or
822 translated proteins thereof at levels that match native stoichiometric levels, temporal dynamics,
823 and *in situ* spatial distributions, this modality is opening new avenues in precision therapeutics.
824 Additionally, the approach leverages the cells existing RNA editing machinery thereby
825 alleviating the need for exogenous and immunogenic proteins to drive editing. In addition to
826 enabling direct repair of G-to-A disease-causing mutations and nonsense mutations, targeted
827 RNA edits can also enable modulation of RNA stability and splicing. Furthermore, transiently
828 modulating protein function, such as the active sites of proteins or modulation of protein-protein
829 interaction interfaces, opens the door to therapeutic avenues ranging from regenerative medicine
830 to oncology. Combined with the intrinsic advantages that RNA-based therapeutics possess of
831 tunability and reversibility and that off targets are non-permanent, these emerging ADAR-based
832 toolsets, coupled with rapidly improving viral and non-viral delivery modalities, are poised to
833 broadly impact biotechnology and therapeutic applications.
834
835

836 **Key words: RNA editing, RNA therapeutics, ADAR, ADAR1, ADAR2, gene therapy, ASO,**
837 **precision medicine**
838
839
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1319 **Figure 1**: Factors affecting ADAR mediated RNA editing

1320 **Figure 2**: Factors influencing gRNA efficiency and selectivity

1321 **Figure 3**: Considerations to support the clinical development of RNA editing

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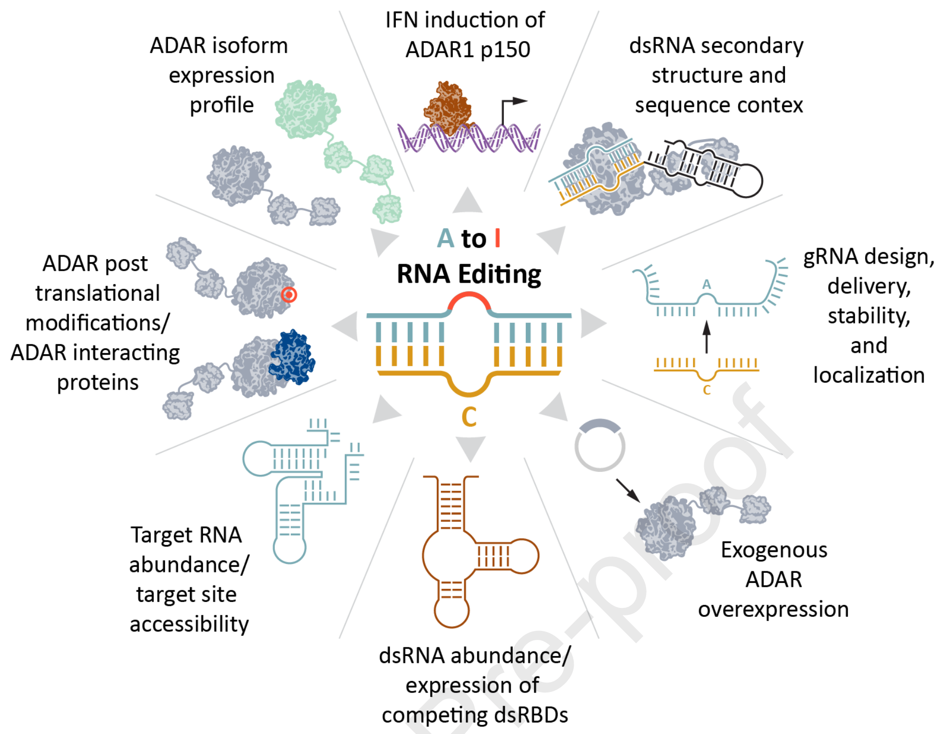

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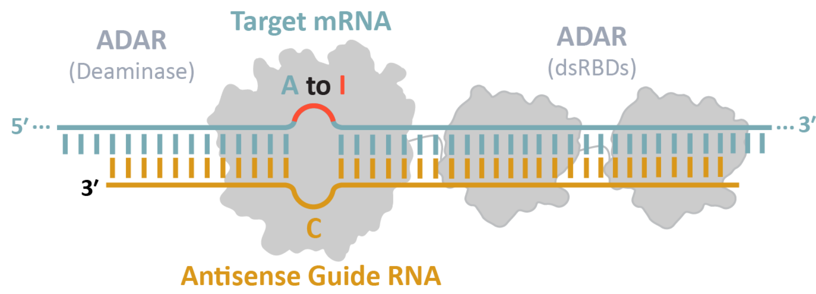

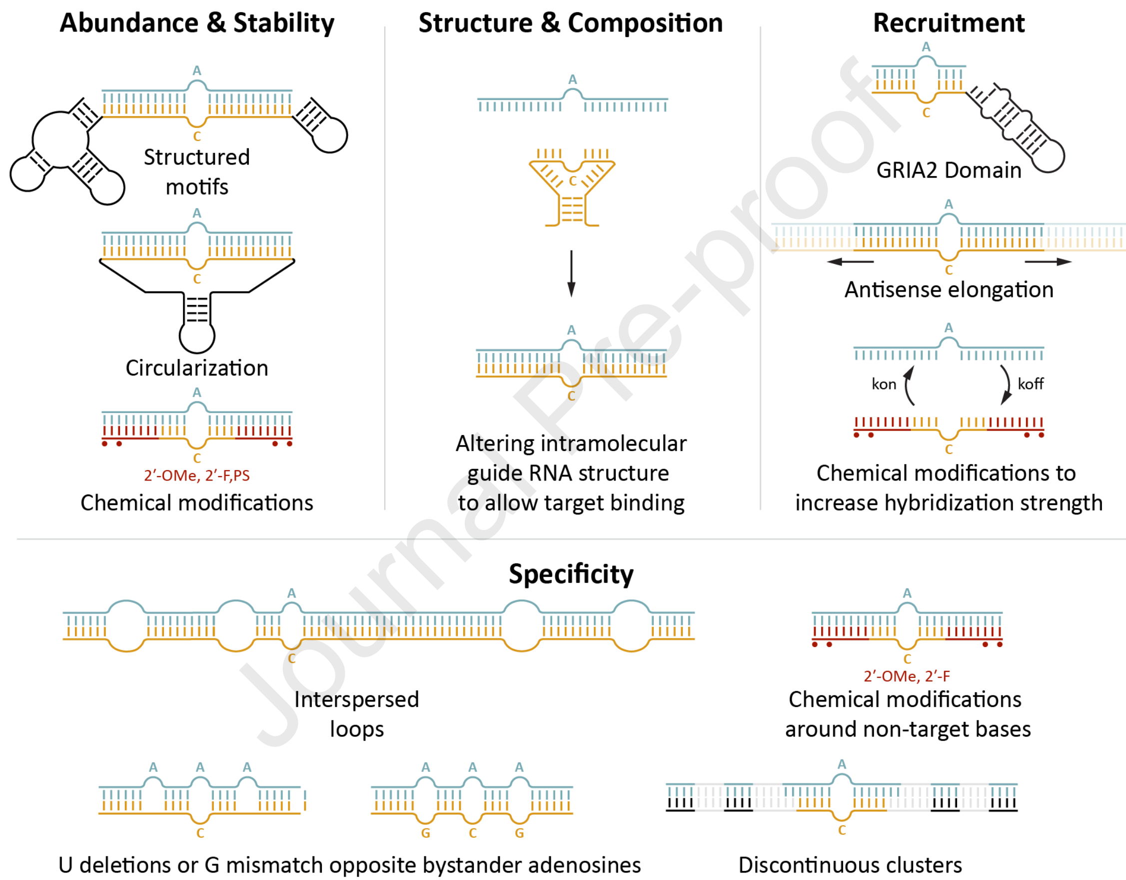

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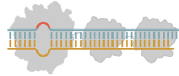
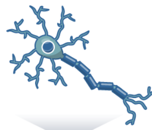
Clinical Considerations:

Molecular



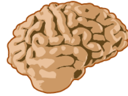
- Percentage of on-target editing and bystander editing
- Transcriptome integrity (i.e., off-target editing, perturbations to expression or splicing, alteration in natural editome)
- Fidelity of cellular machinery to interpret inosine as guanosine

Cellular



- Cellular penetrance and fraction of target cells receiving a therapeutic dose
- Level of protein correction, knockdown, or modulation in target cells
- Impact of delivery modality on immune response and editing efficiency (e.g., type I IFN induction of ADARp150)

Tissue



- Delivery modality of ASO or DNA encoded gRNA for optimal target tissue penetrance
- Biomarkers to indicate restoration of organ health (e.g., glomerular filtration rates, liver enzymes, etc.)

Organism

- Overall pharmacokinetics and biodistribution of the drug
- Adaptive or innate immune response to the payload or to introduced mutationspayload or to introduced mutations
- Clinical endpoints (e.g., cognitive tests for Alzheimer's Disease or mobility tests for muscular dystrophy)

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eTOC Synopsis

ADAR-based RNA editing has emerged as a powerful tool to engineer RNAs, enable correction of disease-causing mutations, and modulate protein functions. We review the emerging field of therapeutic RNA editing, highlight recent laboratory advancements, and discuss the key challenges on the path to clinical development.

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