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Current Strategies for Site-Directed RNA editing using ADARs

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Abstract

Adenosine Deaminases that Act on RNA (ADARs) are a group of enzymes that catalyze the conversion of adenosines (A's) to inosines (I's) in a process known as RNA editing. Though ADARs can act on different types of RNA, editing events in coding regions of mRNA are of particular interest as I's base pair like guanosines (G's). Thus, every A-to-I change catalyzed by ADAR is read as an A-to-G change during translation, potentially altering protein sequence and function. This ability to re-code makes ADAR an attractive therapeutic tool to correct genetic mutations within mRNA. The main challenge in doing so is to re-direct ADAR's catalytic activity towards A's that are not naturally edited, a process termed Site-Directed RNA Editing (SDRE). Recently, a handful of labs have taken up this challenge and two basic strategies have emerged. The first involves redirecting endogenous ADAR to new sites by making editable structures using antisense RNA oligonucleotides. The second also utilizes antisense RNA oligonucleotides, but it uses them as guides to deliver the catalytic domain of engineered ADARs to new sites, much as CRISPR guides deliver Cas nucleases. In fact, despite the intense current focus on CRISPR-Cas9 genome editing, SDRE offers a number of distinct advantages. In the present review we will discuss these strategies in greater detail, focusing on the concepts on which they are based, how they were developed and tested, and their respective advantages and disadvantages. Though the precise and efficient re-direction of ADAR activity still remains a challenge, the systems that are being developed lay the foundation for SDRE as a powerful tool for transient genome editing.

Keywords

RNA Editing; ADAR; Antisense oligo; guide RNA; off-target events

1. Introduction

The purpose of this manuscript is to review on-going efforts to redirect a specific form of RNA editing for therapeutic purposes. To better appreciate the strategies that are being employed, it is useful to give some background on the natural process of RNA editing in question: the site-specific deamination of adenosine (A) to inosine (I), which sometimes recodes genetic information. Adenosine deamination is catalyzed by the Adenosine Deaminases that Act on RNA enzymes (ADARs), which are found in all multicellular

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Conflict of Interest

We declare a competing financial interest. Maria Montiel-Gonzalez and Joshua Rosenthal hold a patent titled: Site-Directed RNA Editing, publication number: US 9,650,627 B1.

metazoans [1-6]. Three genes encoding ADAR-like proteins have been reported in vertebrates, (ADAR1, ADAR2 and ADAR3); however only ADAR1 and ADAR2 are catalytically active [7]. Invertebrates express orthologs to vertebrate ADARS. Thus, RNA editing by adenosine deamination is an evolutionarily conserved mechanism to modify genetic information [8-11].

Although ADARs edit most types of RNAs (mRNAs and miRNAs e.g.), their capacity to recode proteins in mRNAs has received particular interest. When adenosines are converted to inosines at non-synonymous positions within open reading frames, codons are changed because inosines are read as guanosines (G) during translation [12]. Some of the best-studied recoding events affect transcripts encoding the GluR2 subunit of the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor [13]. There are two editing sites in GluR2-encoding mRNAs, the Q/R and R/G sites. Recoding at the Q/R site abolishes Ca^{+2} permeability, while at the R/G site it alters receptor desensitization [14]. ADAR2 knockout mice are not viable because GluR2 Q/R recoding is vital after birth [15]. Interestingly, recoding by ADARs is quite rare in most organisms, but *Drosophila* and cephalopods are notable exceptions [16-18]. Aside from recoding proteins, ADARs also regulate other important cellular functions like the RNAi pathway and the innate immune response [19-23]. Thus, by virtue of its capacity to alter protein and cellular function through recoding or posttranscriptional regulation, ADAR could be used as a potentially versatile and powerful alternative therapeutic.

All ADARs contain two main structural motifs. The first is the double stranded RNA Binding Domain (dsRBD) [24]. The number of dsRBDs differs between ADAR isoforms, as some possess one (>(*C. elegans* Adr1), some two (vertebrate and *Drosophila* ADAR2) and others three (vertebrate ADAR1) [8, 25-28]. Squid ADAR2 has two splice variants, one possessing two dsRBDs and the other three [8]. dsRBDs are ADAR's main targeting domain, recognizing complicated higher order structures within RNAs. They can bind to perfect duplex RNA, though they will also bind to imperfect structures with bulges, hairpins and mismatches with even higher affinity [29-31]. The natural structures that ADARs recognize are diverse. Because no defining motifs have been identified, it hasn't been possible to predict which RNAs will be recognized by ADAR based on their sequence or structural characteristics. Though it is accepted that the main function of dsRBDs is to guide ADAR's catalytic activity to its target adenosine, a better understanding of the recognition mechanisms is vital for the development of ADAR into a molecular therapeutic tool.

The second structural motif, termed the Deaminase Domain (DD), carries out the catalytic activity. The crystal structure of the DD has been solved, and this, coupled with functional studies on ADAR and other nucleotide deaminases [32, 33] have made it clear that ADAR uses a base flipping mechanism to move the adenosine out of the A-form RNA helix, and into the enzyme's catalytic pocket, in order for the A to I conversion to occur [33]. Furthermore, it has been shown that an A-C mismatch at the target A favors editing efficiency. Importantly, crystal structures have hinted that the DD is an independent catalytic unit in the absence of the dsRBDs, and this conjecture has been supported by biochemical studies [32, 33]. For example, work done by the Beal lab has shown that *in vitro* the DD binds with high affinity to ~23 nt long double-stranded RNA in an asymmetric fashion, with

~18 nt on the 5' side and ~5 nt on the 3' side of the editing site [34], although full-length ADAR2 protein usually makes contacts more on the 3' side of the edited A. In addition, the efficiency at which the DD edits a specific target A is highly affected by neighboring bases. For example, these so called nearest neighbor preferences dictate that editing will be efficient when the 5' neighbor is a uracil (U) and poor when it is a G. On the 3' side however, a G is preferred over a U [35]. Editing efficiency can also be manipulated by mutations within the DD. The best example is the E488Q mutation at a position in a loop that occupies the space left in the RNA helix after an A "flipped out" for deamination [36]. The manipulations of both the RNA substrate and the enzyme have been the key tools used by investigators who aim to use ADAR as a molecular therapeutic tool.

It is clear that the overall process of RNA editing by ADARs could be leveraged to correct genetic mutations or fine-tune protein function. Several groups are pioneering work in this area, all of them with the common goal of getting ADARs to edit new targets in a selective way. In recent years genome editing using the CRISPR-Cas9 system has received much attention for the development of therapeutics for a wide variety of diseases. DNA editing offers the advantage of inducing a permanent genetic modification, offering a life-long solution to conditions that are chronic such as Duchenne's Muscular Dystrophy [37, 38]. There are certain instances, however, where RNA editing could be a better alternative than DNA editing. A major of concern associated with Cas9-based genome editing is the potential consequences of off-target events, which can permanently alter genes, disrupting cellular function [39-41]. RNA editing-based therapies could make similar changes in genetic information with the advantage of being transient, eliminating the concern of making permanent alterations. Furthermore, Cas9-based genome editing therapies generally rely on homology directed repair, a process that is only active in dividing cells [42, 43]. By consequence, the use of this approach to alter post-mitotic cells -like neurons- is limited. In contrast, targeted RNA editing can operate in post-mitotic cells, expanding the repertoire of cells that can be treated. This is particularly important for genetic diseases that are diagnosed after the onset of their symptoms in fully-differentiated cells. Another challenge for genome editing is their reliance on large bacterial proteins like Cas nucleases which can trigger immune responses as has been demonstrated by Charlesworth and colleagues [44]. In contrast, RNA based approaches rely on human enzymes that won't be recognized as foreign. Finally, many conditions (e.g. acute pain) are not permanent and therefore a temporary solution is more appropriate. So, how does one deploy RNA editing as a therapeutic tool? Given that most adenosines are not likely to occur in a proper structure for editing, the main challenge is to create an editable structure around the target and redirect ADAR activity to it. We call these new approaches Site-directed RNA Editing (SDRE) and in this review we will discuss the different strategies taken to overcome the challenges in using ADARs as precise therapeutic tools.

2. Site-Directed RNA Editing (SDRE)

The main purpose of the SDRE strategies that we will discuss is to redirect the catalytic activity of ADAR to a target adenosine that is not normally edited and can alter protein function when changed to a guanosine. An extreme example comes with A's in stop codons (UGA, UAA, UAG) which, when edited, allow for read-through during translation; thus,

diseases caused by mutations that introduce termination codon (PTCs) can be corrected by RNA editing. Obviously, most A's do not occur within structures recognized by ADAR; therefore, the strategies that we will discuss try different means to promote the editing of such targets. One way, is to create substrates around a target A that are recognized by ADAR or an engineered ADAR. Essentially, these structures consist of a dsRNA helix surrounding the target sequence, sometimes with a mismatch directly under the A. The simplest strategies use only an RNA oligo to create the dsRNA structure and depend on endogenous ADAR to edit the target A. Other strategies use the RNA oligos as a guiding mechanism for engineered ADARs, replacing their dsRBDs and effectively creating ADAR-derived riboproteins. Based on these approaches, the strategies will be divided into two categories:

- Native ADAR strategies: in which the goal is to redirect endogenously expressed ADAR by specifically recruiting it to a target using an RNA oligonucleotide to create a suitable structure.
- Engineered ADAR strategies: In which the catalytic domain (or DD) of wild type ADAR is engineered to bind a guide RNA (gRNA) that both directs it to a target and creates an editable structure.

Though the goal for both approaches remains the same, each have distinct advantages and disadvantages, which will be discussed in further detail.

2.1. Native ADAR strategies:

The virtue of the native ADAR strategy lies in its simplicity. Relying on endogenously expressed ADAR, it only requires the delivery of an antisense RNA oligonucleotide. In addition, therapeutic antisense-oligos (AOs) have undergone major advances in recent years, including improvements in delivery systems, reductions in toxicity, and improvements in their ability to permeate cells and their stability. This progress has resulted from the ability to modify the oligonucleotides in a myriad of ways through chemical syntheses. An example is Spinraza™, an FDA approved oligo-therapy that promotes exon-skipping, used to treat patients with Spinal Muscular Atrophy (SMA). Aside from producing the desired molecular outcomes, clinical studies have shown that Spinraza is well tolerated by humans, doesn't activate an immune response and a single dose can have a long-lasting effect [45, 46]. In fact, muscle function remains improved up to 85 days after the administration of a single dose. These qualities make the idea of redirecting ADAR through similarly derived AOs extremely attractive.

The first study to use an antisense oligos to redirect endogenous ADAR was published over 20 years ago [47]. The AO design was simple: a perfectly complementary oligo of 25, 34 or 52 nucleotides (nts) centered on the target A. This study demonstrated that the 52 nt long version could promote A to I editing *in vitro*, using nuclear protein extracts of *Xenopus* oocytes as an ADAR source, or *in vivo* when injected into *Xenopus* embryos. This study was the first to show that ADARs can be redirected to correct mutations within mRNAs. However, the experimental design avoided several technical challenges. Mainly, for the *in vivo* experiments, the target mRNA was pre-hybridized to the AO before being injected into the oocytes. This was probably necessary because a perfect RNA duplex, as we now know, is not the highest affinity structure for ADAR recognition and editing [35]. Nonetheless, these

experiments showed the capacity of endogenous ADAR to edit at a structure created by an AO.

Robert Reenan's group was the first to show that endogenously expressed ADAR could be recruited *in vivo* [48]. Interestingly, the intent of this study was not to target a specific adenosine in order to engineer protein function, but rather to create a reporter for ADAR activity in the *Drosophila* nervous system. In previous work, Reenan and colleagues had identified the structures in the pre-mRNA of *Synaptotagmin* (*Syt1*) required to drive RNA editing at two particular sites [49]. Using those structures, the authors developed a GFP reporter that required editing of *Syt1* structural mimics for activation, thereby making it a tool to log the history of ADAR activity [48]. Even though this system was not designed for therapeutic purposes, it did show that with the right structure endogenous ADAR could be recruited to a new target, and it led other groups to the idea that native ADARs could be redirected to edit specific adenosines by mimicking the structures around naturally occurring editing sites.

The Stafforst group was the first to use Reenan's concept of mimicking native RNA structures to redirect wild type ADAR for therapeutic purposes [50]. In this case, they based their AO on a structure that promotes editing at the R/G site of GluR2 [14, 51]. The structure that promotes editing at the R/G site occurs in the pre-mRNA of GluR2, in a region formed by a hairpin between an exon and an intron. This structure is particularly useful because it is small, consisting of only ~40 nts [14, 51]; therefore recreating it using an oligonucleotide, and delivering the oligonucleotide, would be less challenging than with most other structures that are relatively large like the *Drosophila Syt1* structure [48, 49]. The AOs designed by the Stafforst group were mainly composed of 2 parts: i) The 5' region contained the hairpin mimicking the GluR2 R/G structure and ii) the 3' region consisted of 16 nucleotides complementary to the target mRNA, with a C mismatch opposite the target A (Fig. 1). Later versions of the AOs included a 24 nt long hairpin at the 3' end, used to stabilize the guide.

As a proof of principle, their strategy was first tested in Human Embryonic Kidney (HEK)-293T cells by transiently expressing the AO under the control of a U6 promoter. In addition, human ADAR2 and a target gene were co-transfected in separate vectors driven by a CMV promoter. The target was eGFP W58X where a G-to-A substitution introduces a UAG premature termination codon that could be rescued by editing. 48 hours after transfection, correction by editing was determined by fluorescence microscopy and Sanger Sequencing. In these experiments the authors observed editing efficiencies of 40-50%. A further increase to 65% was achieved by integration of ADAR2, under the control of the CMV-tet promoter, into the genome of the HEK-293T cells. In a follow up study, the same group showed that similar AOs lacking the 24 nt long stabilizing hairpin could recruit both the long (p150) and short (p110) isoforms of human ADAR1 [52]. By substituting hADARp150 or hADARp110 into the same assay system used for ADAR2, they reported editing efficiencies of ~37% and 20%, respectively. Though this system is still in development, the Stafforst group has successfully shown that both ADAR1 and 2 can be redirected to edit a PTC by using RNA oligonucleotides that mimic structures naturally recognized by ADAR.

Soon after these publications, the group of Nakagawa developed a similar system based on the same GluR2 R/G structure [53]. In this case the gRNA had a smaller region complementary to the target (9 nucleotides instead of 16). Another difference was that the R/G hairpin structure was closer to the target A. This gRNA also lacked the stabilizing hairpin at the 3' end. The authors showed that having the complementarity region on the 5' side of the R/G structure instead of the 3' side resulted in higher editing efficiencies when testing the system using eGFP W58X correction. Overall, this design resulted in a n editing efficiency of 28-32% in HEK-293 cells.

Both the Stafforst and Nakagawa groups have shown that mimicking native RNA structures can promote editing of an Amber stop codon (UAG) by heterologously expressed ADAR. An important next step will be to recruit endogenously expressed ADAR. In the studies cited above, ADAR was over-expressed (transiently or genomically) under the relatively strong CMV promoter; endogenous ADAR expression levels are certainly far lower in most tissues. Furthermore, it will be useful to have a direct comparison of editing efficiency between these structural mimics and the simple, perfect duplex design originally used by Woolf [47]. Mimicking natural structures is tricky and cannot be complete; there can be no perfect mimics because there must always be a region that is complementary to the target A which is dictated by the surrounding sequence and is not part of the naturally occurring blueprint. Another challenge relates to the fact that ADAR is not freely accessible in the cytoplasm [54]. Most ADARs are concentrated within the nucleolus although small amounts must be present in the greater nucleoplasm to carry-out editing at endogenous sites [54]. The long form of ADAR1 is the exception as it shuttles between the nucleus and the cytoplasm following induction by interferon [55]. Thus, the effective recruitment of endogenous ADAR is not simple and may benefit from targeting the AO. Nevertheless, no matter what eventually turns out to be the most effective oligonucleotide design, these systems hold much promise, and the development of guides that can effectively recruit endogenously expressed ADARs will be a big step forward.

2.2. Engineered ADAR strategies:

In contrast to the oligo-only strategies discussed in the previous section, the use of an engineered ADAR for SDRE has the major advantage of not depending on the expression levels of endogenous ADAR. Unlike native ADARs, which are normally sequestered within the nucleolus [54], engineered ADARs can be routed to the cytoplasm to interact with mature messages. Furthermore, with engineered ADAR strategies, the activity of ADAR itself can be rationally redesigned, making it customizable to meet specific needs. All the strategies that have been developed are based on the fact that the DD of ADAR is an independent catalytic unit, having activity in the absence of the dsRBDs [32] This has enabled the use of an alternative guiding mechanism to the dsRBDs. Indeed, all strategies use an antisense RNA oligonucleotide as a guide (gRNA). The approaches differ in the method used to couple the guide RNAs to the DD. A weakness in these strategies lies in the fact that two components (gRNA and engineered ADAR) must be delivered, making clinical applications more challenging. In particular the delivery of the enzyme (genetically encoded or not) will be a big hurdle to overcome, particularly if it is perceived as foreign and

provokes an immune reaction. Nonetheless, there have been some promising preliminary results.

2.2.1. SNAP-tag—The SNAP-tag reaction is one approach that has been used to couple a gRNA to the DD of ADAR. The SNAP-tag system was originally developed to covalently label fusion proteins *in vivo* by the Johnson lab [56]. The system relies on the O⁶-alkylguanine-DNA Alkyltransferase (hAGT) protein that catalyzes the irreversible transfer of an alkyl group from O⁶-alkylguanine to a cysteine residue in the same enzyme. Interestingly, hAGT can transfer any functional group or molecule attached to the substrate, which can also be O-Benzylguanine (BG). Using this system, the Johnson lab successfully proved that a mutated form of hAGT (later called a SNAP-tag) could be tagged with BG-fluorescein *in cellula* in Chinese Hamster Ovarian cells. The Stafforst group adapted this reaction system and used a SNAP-tag fused to the N-terminus of ADAR's DD to link it to the gRNA [57]. For their system, they created SNAP-tag hADAR1 DD or hADAR2 DD fusion proteins, in which the SNAP-tag had been mutated to have higher activity against BGs substrates [56, 58]. These constructs were then subcloned into a mammalian expression vector under the control of the CMV promoter. Thus, when expressed in cells, the hATG domain catalyzes the transfer of the gRNA from synthetic BG-gRNA the SNAP-tag DD fusion protein (Fig 2).

Once the gRNA is bound to the DD, it can guide it to the mRNA target. The gRNAs were 17 nts long perfectly matching AOs, except for a single C mismatch under the target A and positioned in the middle of the gRNA (Fig 2). BG-gRNAs were synthesized *in vitro* by reacting pre-activated BG-linker-COOH with chemically synthesized NH₂-gRNAs. The formation of the BG-gRNA conjugate was confirmed by Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry. Also, for stabilization, the gRNAs had 2'-O-methyl modifications, as these backbone modifications make them more resistant against nucleases [59]. Interestingly, the authors showed that 2'-O-methylation on the C opposite to the target A strongly inhibits editing, while the same modification in all the bases in the gRNA except for the three nucleotides in the middle opposite to the target, did not affect editing efficiency.

For the first test of the system, HEK-293T cells were transfected with the DD-SNAP-tag plasmid and with the target sequence in a CMV-driven vector. The target used was the enhanced Cyan Fluorescent Protein (eCFP W66X) containing an UAG PTC, similar to the eGFPW58X codon described in the Native ADAR strategies. 24 hours post-transfection the cells were re-plated and allowed to settle for 24 hours more before being transfected with BG-gRNA. Editing was assessed 24hrs afterwards by fluorescence microscopy and Sanger Sequencing. With these experimental conditions, the authors reported an editing efficiency that resulted in ~30% correction [59]. Further tests of the system showed that the SNAP-tag DD can also edit UAG triplets in endogenously expressed targets in the HEK-293 cells (GAPDH, ACTB e.g.) with efficiencies up to ~90%, depending on which version of the DD is used [60]. Though they found there was no significant difference in editing efficiencies between DDs from hADAR1 and hADAR2, when the DD contained the E488Q mutation (that increases the catalytic activity of ADAR as previously discussed) they saw a general increase in editing efficiencies, in particular on targets that were poorly edited with the wild

type DDs. Though the editing efficiencies on target A's were high, the authors also observed off-target edits that will be discussed later in this review. The authors also showed that their system can edit adenosines with a variety of 5' neighbors (NAG) *in vitro* [61], since the UAG codon extensively tested *in cellula* is in an optimal context for editing according to the nearest neighbor preferences of ADARs [35]. Extending the system's efficiency to different codons *in cellula* and *in vivo* will be an important step on the road to applying it in clinical settings.

A particularly attractive aspect of this strategy is that chemical modifications (e.i. 2'-*O*-methyl) can be easily introduced to the gRNA to provide stability and manipulate editing efficiency on target and off-target A's. This could translate into longer lasting effects when used for therapeutic treatments, particularly considering how delivery systems for AOs have improved over the last years. On the other hand, the fact that the gRNAs cannot be genetically encoded puts limitations on delivery systems and increases costs.

2.2.2. λ N-BoxB—Another strategy to link ADAR's DD to a gRNA is by using the Lambda N protein (λ N) and the BoxB RNA hairpin, a protein-RNA interaction that naturally occurs in the bacteriophage lambda. The λ N protein is small (12.2 kDa) and serves as an anti-terminator of the transcription of bacteriophage genes. By binding to nascent messages, it forms a complex with the RNA polymerase, allowing for all the genes of the bacteriophage to be transcribed [62]. λ N's binding sites are 15-17 nt stem-loops, called BoxB's. Structural studies have shown that a 22 amino acid peptide of λ N is sufficient for BoxB binding; both the 22 amino acid peptide and the full-length N proteins bind to BoxB with similarly high affinity ($\sim 10^{-8}$ M). The small size of the λ N peptide and BoxB hairpin, and the high affinity of their interaction, made them an attractive system to couple the hADAR2 DD to gRNAs. To do so, the λ N peptide was fused to the N-terminus of hADAR2 DD (λ N-DD) and a repeat of the λ N unit 4 times in tandem (4 λ N-DD) was found to be most effective [63, 64]. As with the SNAP-tag system, the E488Q mutation can also be introduced to the DD. Just as multiple λ N peptides were added to the DD, two BoxB hairpins were added to the gRNA (Fig 3). To promote target recognition and DD binding, the gRNAs were composed of three regions: i) a central region of 29 nt fully complementary to the target mRNA except for a C mismatch under the target A, ii) two 17 nt long BoxB RNA hairpins, one located 11 nt on the 5' of the target A and one 19 nt to the 3' and iii) 2 fully complementary regions of 10 nt on each terminus of the guide to act as anchors (Fig 3). The gRNA sequences were cloned into a small RNA expression vector which is driven by a U6 promoter [65].

To test the system, HEK-293T cells were transfected with the vectors expressing the 4 λ NDD enzyme, the gRNA and the target sequence. As a target, an mCherry-eGFP (W58X) dual reporter system was used to allow a better quantification of editing efficiencies in relationship to transfection efficiency. Correction of PTCs in a variety of neighboring contexts were tested (besides the highly editable Amber codon UAG, all combinations of UGAN and UAAN were also tested). Editing was assessed 4 days post-transfection by both imaging fluorescence and Sanger Sequencing. Editing efficiencies, reported as eGFP correction, ranged from 20% to 70% depending on the sequence around the target

adenosine. In general, the E488Q increased on-target efficiencies (~40% increment) in comparison to the wild type λ NDD, but this was highly context dependent.

In contrast to the SNAP-tag system, the gRNAs for the λ N-DD system can be genetically encoded and thus can be delivered as plasmids or viruses. The disadvantage is that the gRNAs are less stable and must be delivered in higher quantities. As described later (see off-target editing section), high gRNA expression can lead to non-specific editing. Nonetheless, the Mandel group showed that the λ NDD-BoxB system can be delivered in a single Adeno-Associated Virus (AAV1/2) [66]. Furthermore, they could be delivered into cultured primary mouse hippocampal neurons and correct an A/G mutation in the Methyl CpG Binding Protein 2 mRNAs (MECP2) with an efficiency of ~75% [66]. These results are very promising as they prove that the λ NDD-BoxB system works on endogenously expressed messages and they suggest that the strategy could eventually be delivered to cells *in vivo*, where tissue specific promoters could drive selective expression.

2.2.3. MS2—A strategy very similar to the λ NDD-BoxB system employs the MS2 protein, another bacteriophage protein that recognizes RNA hairpins. MS2 is a 13.7 kDa coat protein from group I *E. Coli* bacteriophages that functions both as a transcriptional repressor and a structural protein [62]. For its function as a transcriptional repressor *in vivo*, MS2 forms homodimers in order to bind to RNA. Similar to the λ N protein, MS2 recognizes hairpins with a loop sequence AUUA with high affinity (~10⁻⁹ M). Studies trying to determine how the loop sequence regulates MS2 binding, discovered that loop sequence AUCA increases affinity of MS2 (~10⁻¹⁰ M). Furthermore, an unpaired adenosine two nucleotides before the loop on the 5' arm of the stem is also necessary for high MS2 affinity for RNA [62]. Given the high affinity of MS2 protein for the target RNA, the Tsukahara group used it to bind the gRNAs [67]. The DD of *hADAR1* was fused downstream of MS2 and expression was driven by the strong CMV-IE94 promoter [68]. Thus, in this engineered protein, the MS2 protein binds stem-AUCA loops engineered into the gRNAs (Fig 4).

The gRNAs for this particular strategy have two parts: The 3' region consists of 21nt that are fully complementary to the target mRNA, with the target A located in the middle. As before, a C mismatch with the target was used to favor editing. In some cases, a G mismatch was used to inhibit editing at off-target sites (Fig. 4). The 5' region consists of 6X tandem repeats of the stem-AUCA loop RNA sequences. gRNA expression is driven by an RNA Pol II promoter (CMV-IE94) instead of an RNA Pol III promoter for undisclosed reasons.

As with the previous strategies, the system was tested in HEK-293 cells. Vectors driving either the gRNA, the MS2-ADAR1DD fusion protein or the target (eGFP W58X; UAG) were transfected and editing efficiency was evaluated by fluorescent microscopy and Sanger Sequencing. With this approach, the authors reported an editing efficiency of 5% [67]. This low editing efficiency was somewhat surprising given that the MS2 binds to its hairpin target with a higher affinity than does λ N. There could be many contributing factors. *In vivo* dimerization of MS2 is needed for binding [62], and MS2-ADAR1DD fusion proteins may need to form dimers before binding the gRNA. Furthermore, the use of the RNA Pol II promoter for the expression of the gRNA could have diminished expression or promoted

gRNA modification (e.g. capping or polyadenylation). Accordingly, this system needs to be characterized more thoroughly.

2.2.4 CRISPR-Cas13b—In recent years, the use of Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated Nuclease Cas9 system to edit genomes has gained wide popularity due to its efficiency and specificity [69]. Interestingly, similar to the SDRE strategies discussed thus far, CRISPR-Cas systems also use gRNAs to direct the nuclease to a target DNA. However, in contrast to the SDRE strategies that rely on naked RNA to recognize and hybridize to the target sequence through basic Watson-Crick base-pairing, Cas proteins envelope much of the gRNA, providing protection and orientation [70]. Though the CRISPR-Cas9 system was developed for gRNA directed DNA editing, not all CRISPR associated nucleases are DNases. In particular Cas13 is a type VI CRISPR-associated RNase. The Zhang lab decided to take advantage of the CRISPR-Cas system and adapt it to RNA editing using Cas13 [71].

So far, there are four Cas13's that have been identified: Cas13a, b, c and d [70]. Each contains two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains that mediate RNA cleavage. In order to adapt the CRISPR-Cas system for SDRE, the Zhang lab made two histidine to alanine substitutions (H133A/H1058A) within the HEPNs of Cas13b of *Prevotella sp.* (which showed high efficiency RNA cleavage in mammalian cells) to eliminate its RNA-nuclease activity [71]. This mutated version of Cas13b (dCas13b) was then fused at the N terminus of to the DD of ADAR1 or 2 to serve as the auxiliary structure to bind gRNAs and target. To increase the catalytic efficiency of the fusion enzyme, the authors also included the hyper-activating mutations E1008Q for ADAR1 and E488Q for ADAR2 (the two mutations are at analogous positions). Cas nucleases typically bind to secondary RNA structures formed by the Short Palindromic Repeats (also called Direct Repeats) sequences [69, 70]. In this case, dCas13 specifically binds to the hairpin with a bulge formed by a 36 nt standard Direct Repeat that the Zhang lab have engineered into their gRNAs (Fig. 5).

The gRNAs are composed of two main parts: at the 5' there is a 50nt long region complementary to the target sequence, that the authors call the "Spacer" to keep with the nomenclature developed for other CRISPR-Cas systems. As with other strategies, the gRNAs were designed to have a C mismatch opposite to the target A. At the 3' part of the gRNA there is a 36 nt hairpin-forming standard Direct Repeat sequence recognized by dCas13b (Fig 5). Several locations of the C mismatch were tested while establishing the system. In general, a position ~34 nt upstream of the 3' part was found to be optimal. For each target tested, the "Spacer" was subcloned downstream of the standard Direct Repeat for dCas13b and expression of the gRNAs was driven by a U6 promoter, similar to the λ NDD-BoxB system.

To assess the strategy in mammalian cells, the authors transfected HEK-293FT with the dCas13-DD fusion vector, the gRNA vector and the vector containing the target gene (*Cypridina* Luciferase (CLuc) W85X; UAG). Furthermore, the authors tested every possible combination of three nucleotides with an A in the middle in the same W85 position of CLuc. Editing efficiency was assessed 48 hours after transfection by measuring luciferase activity

and by Illumina sequencing. Correction of the Amber codon was also tested in two disease-related mutated genes: i) X-linked Nephrogenic diabetes insipidus (*AVPR2* W293X) and ii) Fanconi anemia (*FANCC* W506X); as well as in another 34 genes with disease-related G to A mutations. Overall, for all the targets tested the authors observed editing efficiencies up to ~30% [71].

This system is of particular interest due to the widespread use of the CRISPR-Cas9 systems [72, 73]. As CRISPR based systems receive enormous focus, it is likely that many tools being developed for them will be applicable to Cas13b as well. The fact that the strategy was tested on a great array of targets (around 52) in comparison to the few targets tested by the SNAP-tag or the λ N-BoxB system (Table 1), hints at the utility of this system. Thus, the rapid development of CRISPR-Cas technologies combined with the fact that this strategy produces editing efficiencies similar to those observed with the SNAP-tag and λ N-BoxB systems makes Cas13b an attractive option for SDRE. However, as with CRISPR-Cas9, the requirement to deliver Cas13b for SDRE is a major drawback for most clinical applications. Cas13 is 110 kDa and coupled to the DD (~40 kDa), the fusion enzyme turns into a big protein for delivery, especially in comparison to the λ N-BoxB or the MS2 strategy where the total size of the fusion protein is less than 60 kDa. Furthermore, as Cas13b is a bacterial protein, there is a significant chance that it will provoke an immune response. This is also true for λ N and the MS2, but they are much smaller entities. In fact, the toxicities of all systems need to be better studied before any can progress to clinical settings.

2.3. Off-Target RNA editing with the different SDRE approaches

All off the engineered ADAR strategies discussed thus far are effective at editing specific A's in different mRNA targets and within varied nucleotide contexts; however, a drawback for all of them is that the activity of the fused enzyme is not completely specific, generating off-target edits. We classify these events into four categories:

- i. Off-targets in the target mRNA within the region complementary to the gRNA
- ii. Off-targets within the target mRNA outside of the region complementary to the gRNA
- iii. Off-targets within the gRNA
- iv. Off-targets in non-targeted mRNAs (transcriptome-wide off-targets)

Gauging off-target events is important because they raise concerns about the safety of these strategies for therapy. It is possible that such off-targets could lead to deleterious functional changes in both the targeted and non-targeted proteins. Furthermore, because the gRNA itself can be edited, this could cause further off-target editing by changing its specificity.

Therefore, the labs working on these different strategies have tried different approaches to diminish off-target editing.

2.3.1. SNAP-tag—The Stafforst group used two different approaches to diminish off-target effects. The first approach takes advantage of the ease with which chemical modifications (2'-*O*-methyl e.g.) can be introduced to the gRNAs. They report that off-

targets in the region under the gRNA (type I) can be reduced by adding 2' methoxy or 2' fluoro modifications to the gRNA in the nucleotides opposite to the off-target A's. The inclusion of such modification limited off-target editing of a CAA codon in endogenous GAPDH to 20% when using transiently expressed SNAP-tag DD containing the E488Q mutation [60]. Though chemical modifications of the guides seemed effective in reducing type I off-target effects, this approach would not work with types II and IV. The second approach aimed to reduce type II and IV off-target events by integrating the coding DNA for the SNAP-tag DD into the genome of HEK-293T cells. With this approach the authors observed 6 and 30 off-target events with the SNAP-tag DD from hADAR1 and 2 respectively. In contrast they reported 835 off-targets (quantified using RNAseq) when cells expressed the hADAR1 DD E488Q enzyme, and 1310 when they expressed the hADAR2 DD E488Q enzyme. Importantly, out of the 835 and 1310 off-targets observed, about 50% were located within 3'UTR regions [60]. Edits in this region are not necessarily benign because the 3'UTR has important roles in post-transcriptional regulation and translation [74]. Though the authors showed that integration of the fusion enzyme DNA into the cell genome reduces off-target events, the implementation of this solution would prove difficult for therapeutic purposes for transient correction since it would require altering the patient's genome.

2.3.2. λ N-BoxB—With the λ NDD-BoxB strategy ~77,000 off-target sites all over the transcriptome were noticed when using the λ NDD version containing the E488Q mutation [75]. Similar to the previous strategy, for the λ NDD-BoxB strategy two approaches have been tested to diminish off-target events. The first approach takes advantage of the fact that G to A mismatches in general are known to decrease editing efficiencies [76]. When G mismatches were placed in the gRNAs under non-targeted A's, type I and II off-target events could be diminished [75]. However, type II and IV off-target events were still numerous, particularly in the presence of the E488Q version of the λ NDD enzyme. It was then hypothesized that type II and IV off-target events could be reduced if the time that the enzyme interacts with non-targeted messages was somehow reduced. Using the standard system, both the gRNA and the enzyme would be mostly expressed in the cytoplasm, along with the bulk of mature mRNAs. In order to restrict the enzyme's interaction with non-targeted mRNAs, a Nuclear Localization Signal was appended to its N-terminus, thus diminishing type IV off-targets by 50% [75]. It is important to keep in mind that though the rates of off-target events reported for this strategy remain at least 8 fold higher in comparison to other strategies (Table 1), a direct comparison between the number of off target events reported cannot be drawn. Differences in reported off target events are highly dependent on the depth of the sequencing, the pipeline of data analysis and the editing cut-off value. Interestingly, the frequency of off-target edits correlated much more closely with gRNA expression than with enzyme expression [75]. Therefore, improvements to the system that enable the delivery of less gRNA would be expected to reduce off-target edits.

2.3.3. CRISPR-Cas13b—For the CRISPR-Cas13b strategy the Zheng lab used one approach to reduce off-target events. Initially, the authors reported ~18,835 off-target events. They hypothesized that this number could be decreased by destabilizing the RNA-binding capacity of the hADAR2 DD-E488Q. In order to find a version of hADAR2 DD-E488Q

with a reduced affinity for dsRNA, they mutated critical residues important for RNA binding within the DD (R348E, V351L, T375S, T375G, R455E, R455G, N473D, R510E, T490A) [71]. Results of transcriptome-wide RNAseq demonstrated that E488Q/T375G mutant had the highest editing efficiency with the lowest number of transcriptome-wide off-target events, reducing them to 20. Also, this mutant improved editing efficiency from 13 to 27% on endogenous genes with no detectable type I or II off-target edits. Interestingly, the group of Stafforst also tried the E488Q/T375G mutant on their SNAP-tag system and, though they did see reduction in off-targets, they also observed an overall decrease in editing efficiency. Nonetheless, extra modifications of ADAR's DD seems to be an effective way to reduce off targets, further demonstrating the versatility of ADAR as it can be engineering to meet specific needs.

Although further ADAR engineering and modifications of gRNAs seem to be valid approaches, off-target events are still one the major drawbacks for the clinical application of SDRE strategies. At present it appears that the SNAP-tag ADAR system leads to the fewest off-target edits, while still maintain robust on-target editing. A direct comparison of the extent of off-target editing between all the systems is difficult at present because reported values were based on different analysis pipelines with different RNAseq coverage and different editing thresholds for calling off-target events. While the presence of off targets is not ideal, the fact that RNA is transient makes it likely that they would not be as dangerous as those within DNA because they would not cause permanent changes. Nonetheless better strategies to completely eliminate off-target events by SDRE systems are merited. Since both, the enzyme and the gRNA can be co-engineered in all the engineered enzyme strategies, one could theorize that it would be possible to co-evolve target-specific gRNA-ADAR pairs that give high editing efficiency and specificity.

3. Conclusions and future directions

Overall, the strategies that have been discussed show that SDRE has the potential to be used as a therapeutic alternative for the treatment of many diseases. Although each one uses its own particular approach, they all show that it is possible to correct mutations within mRNAs; in particular PTCs (Table 1) associated with various diseases such as Cystic Fibrosis [64] or early onset of Parkinson Disease [50] are attractive targets. Moreover, aside from nonsense mutations, some strategies were also tested in the context of missense mutations (Table 1) associated with diseases such as the Rett Syndrome [66] or the Factor V Leiden Thrombophilia [59]. In most cases, the pathologies that result from these and other mutations are extraordinarily challenging to treat by conventional means and normally focus only on the symptoms. With SDRE as an alternative, all that is needed is an editing enzyme (endogenous or engineered) and a gRNA. Such simplicity, shared with CRISPR-Cas9-based genome editing, makes SDRE an attractive approach for the treatment of genetic diseases.

However, in contrast to CRISPR-Cas9-based approaches, SDRE has the potential to be used for temporary conditions that are not associated with genetic mutations. Though none of the discussed strategies has explored this avenue, it is possible to think of SDRE as a tool to fine-tune protein function in biological processes that become dysregulated in response to inflammation or injury. For example, inflammation can lead to the development of

Myasthenia Gravis, where nerve-muscle communication functions improperly, or chronic pain, where nociception is altered [77-79]. SDRE could potentially be used to transiently modify the properties of neurotransmitter receptors or ion channels involved in these processes to re-establish normal function without causing long lasting genomic changes. A major advantage that SDRE has over CRISPR-Cas9-based genome editing is its potential use to treat non-genetic conditions in post-mitotic cells.

Although each SDRE approach has its advantages and disadvantages, we have yet to arrive at the optimal system. The knowledge acquired in the development of these technologies, coupled with the indispensable basic studies on the structure of ADARs and the molecular biology of RNA editing, have been instrumental in moving this field forward. At present, there are relative few laboratories around the world pioneering these approaches. That will surely change because the therapeutic potential of SDRE is on par with genome editing. The main challenges lie in increasing editing efficiency on difficult targets, developing efficient mechanisms for delivery, reducing or eliminating off-target events, and obscuring the systems from detection by the immune system. These challenges are not insurmountable. Progress in SDRE is moving at a rapid pace and it is reasonable to postulate that the technology will be ready for clinical application in the not too distant future.

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Highlights

- Several strategies have been developed to redirect the catalytic activity of Adenosine Deaminase Acting on RNA (ADAR) in order to correct G to A genetic mutations and engineer protein function.
- One approach is to mimic naturally occurring RNA structures to recruit endogenous ADAR to specific target mRNAs.
- Another approach is to couple the catalytic domain of ADAR to a guide RNA that serves to direct the engineered enzyme to the target mRNA.
- Though all the strategies can successfully edit target mRNAs, they all must be further developed to overcome drawbacks before they can be applied in a clinical setting.

Figure 1. Recruiting endogenous ADAR.

Endogenous ADAR1 or 2 is recruited by mimicking native RNA structures. Antisense RNA oligo containing a C mismatch is composed mainly of two parts: 3' region complimentary to the target mRNA (in green) and a 5' region that forms the secondary structure resembling the endogenous GluR2 R/G target sequence (in yellow). This structure is recognized by ADAR's dsRBM (in orange) that guide the DD (in red) to the target A under a C mismatch.

Figure 2. SNAP-tag Strategy.

The SNAP-tag enzyme (in orange) covalently binds to the gRNA (in green) through a linker region (in magenta). The gRNA then brings the DD (in red) to a specific region of a message and also creates a suitable dsRNA substrate surrounding the targeted A. The gRNA contains a C mismatch at the target A to promote more efficient editing.

Figure 3. λN-BoxB strategy.

The editing enzyme containing the λN peptides (λNx4, in orange) fused to the ADAR2 deaminase domain (in red) is guided to the target mRNA by the gRNA (in green). This gRNA is composed of three regions: i) a central region fully complementary to the target mRNA except for a C mismatch at the target A, ii) two BoxB RNA hairpins positioned strategically to promote efficient editing and, iii) two fully complementary regions on each terminus of the gRNA to stabilize it.

Figure 4. MS2 strategy.

The gRNA consists of two parts: The 5' region fully complementary to the target mRNA (in purple). At the target A, there is C mismatch to favor editing, while a G mismatch is used to reduce editing in non-target As. The 3' region consists of a 6X tandem RNA stem-AUCA loop repetition that is recognized by the MS2 protein (in orange) fused to the DD of human ADAR1 (in red).

Figure 5. CRISPR-Cas13b Strategy.

The gRNA (in green) is composed of a 50 nt long 5' region complementary to the target sequence, with a C mismatch opposite to the target A. The 3' region of the gRNA is a 36 nt hairpin-forming the Direct Repeat recognized by Cas13b. Catalytically dead Cas13b (DCas13b in orange) fused to the DD of human ADAR1 or 2 (in red) binds to the Direct Repeat hairpin and positions the DD of ADAR1/2 in the vicinity of the target A on a mRNA (in purple).

Table 1.

Summary and comparison of the main aspects of Site-directed RNA editing strategies discussed.

Method	Enzyme	Reporter targets	Disease-related targets	Editing efficiency % ranges in cellula	Context tested	Transcriptome-wide off targets
<u><i>Endogenous ADAR</i></u>	ADAR1 ADAR2	eGFP(W58X)	PINK1(W437X)	20-37	UAG	Not tested
<u><i>SNAP-tag</i></u>	DD hADAR1 DD hADAR2	eCFP(W66X)	Factor V Leiden polymorphism (R534Q <i>in vitro</i>)	30	All possible combinations	6 to 1,310
<u><i>AN-BoxB</i></u>	DD hADAR2	mCherry-eGFP(W58X)	CFTR(W496X) MECP2 (W104X, R106Q, R306H)	20-70	All possible combinations	~8,800
<u><i>MS2</i></u>	DD hADAR1	eGFP(W58X)	None	5	UAG	Not tested
<u><i>CRISPR-Cas13</i></u>	DD hADAR1 DD hADAR2	Cypridinia Luciferase (W85X)	AVPR2 (W293X) FANCC (W506X) and 33 more	23-35	All possible combinations	20