RNA Repair

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An RNA–Deaminase Conjugate Selectively Repairs Point Mutations**

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RNA modification is an important mechanism in higher organisms to regulate gene expression and to diversify their gene products.[1–3] Typical modifications include pseudo-uridinylation, 2′-hydroxymethylation, and adenosine-to-inosine (A-to-I) editing and are abundantly found in tRNA and rRNA. In contrast to other modifications, the effect of A-to-I editing is readily predictable since inosine is prevalently read as guanosine in biochemical reactions. Thus, A-to-I editing formally introduces an A-to-G point mutation on the RNA level and results in, for example, 1) the highly specific reprogramming of single amino acid codons, and 2) the alteration of RNA splice patterns by forming or destroying splice elements.[1,2] Furthermore, A-to-I editing alters RNA interference[4] by targeting primary micro-RNAs (pri-miRNAs),[5,6] and finally, hyperediting seems involved in viral defense.[1] Physiologically, A-to-I editing is essential for the survival of higher organisms, as exemplified by the site-selective editing of the glutamate receptor.[1,2] A more recent example demonstrates how A-to-I editing supports the adaptation to environmental conditions.[7] Consulting the table of the genetic code one finds that 12 out of the 20 canonical amino acids could be targeted including Asp, Glu, Asn, Gln, His, Lys, Arg, Ser, Thr, Tyr, Ile, and Met/Start, and all three Stop codons, a striking accumulation of residues that are essential for enzyme catalysis, posttranslational modification (signaling), and protein function in general. Consequently, harnessing enzymatic A-to-I deamination would make it possible to manipulate RNAs and their protein products in a currently unprecedented manner.

Examples from the literature demonstrate the feasibility of redirecting pseudo-uridinylation[8] and 2′-hydroxymethylation[9] activity to alter gene expression. However, such enzymes are naturally directed to their RNA substrates by small guide RNAs (gRNAs) which makes it particularly easy to steer their editing activities towards new targets by simply reprogramming the respective gRNA components.[10] Unfortunately, the ubiquitously expressed adenosine deaminases acting on RNA (ADARs) are not riboproteins. Instead they bind their reaction partners with N-terminally fused double-stranded (ds) RNA-binding domains.[1,2] Another obstacle: the dsRNA secondary structure that is the prerequisite for reaction must be self-contained in the target RNA. Thus, it seems unfeasible to direct ADAR enzymes for a specific reaction at a new substrate. However, applying simple Watson–Crick binding rules for targeting is most rational and desirable. To turn hADAR1 into a gRNA-dependent enzyme, we thus fused the isolated C-terminal deaminase domain (aa 798–1226) of hADAR1 to the C-terminus of a SNAP-tag[11] domain (an engineered O^6-alkylguanine-DNA-alkyl transferase), thereby removing the natural substrate-binding domains of hADAR1 (aa 1–797, Figure 1 A). The corresponding fusion protein was expressed and purified in an adapted literature protocol (see the Supporting Information).[12] The chemoselective reaction of the SNAP-tag with 5′-*O*-benzylguanine (BG)-modified gRNA was then applied to generate covalent gRNA–deaminase conjugates (Figure 1 B; for the synthesis of BG-gRNA see the Supporting Information).[11] As expected, conjugates were quantitatively formed within 30–60 min at a probe concentration of roughly 1 μM (Figure 1 C) and could be purified by heparin ion exchange if desired. The UV absorption spectra of the purified conjugate overlaps well with the sum of its two components, SNAP–deaminase and gRNA (Figure 1 D). Lacking the natural substrate-binding domains, the gRNA component of the conjugate fulfills two essential tasks: first, substrate binding, and second, formation of the secondary-structure motif that is required for efficient editing.

We first studied the repair of a $Stop^{66}$ (U<u>A</u>G)→Trp^{66} (U<u>G</u>G) nonsense mutation in the open reading frame (ORF) of the gene of the enhanced-fluorescent protein (eCFP) (Figure 2 A).[13] This gene was selected for two reasons. First, editing is reported by a positive fluorescence readout, and second, the $Stop^{66}$ codon is surrounded by an adenosine-free patch of mRNA which prevents unwanted overediting of neighboring adenosines. Consulting the literature, we found that a $\geq$15 base pair (bp) dsRNA duplex containing a central A/C mismatch was reported as the minimal motif for efficient editing, notably, with the mismatched adenosine as the exclusive target.[14] Thus, a 17 nucleotide (nt) gRNA was designed that places the targeted adenosine (U<u>A</u>G) centrally into the desired environment. To simplify the procedure, conjugation was performed in situ by adding SNAP–deaminase and BG-gRNA together to the reaction, with the protein in 1.3 molar excess relative to gRNA to enforce full conversion of the latter. Reactions were carried out at mRNA concentrations ranging from 500 nM down to 10 nM, roughly covering the concentration range of

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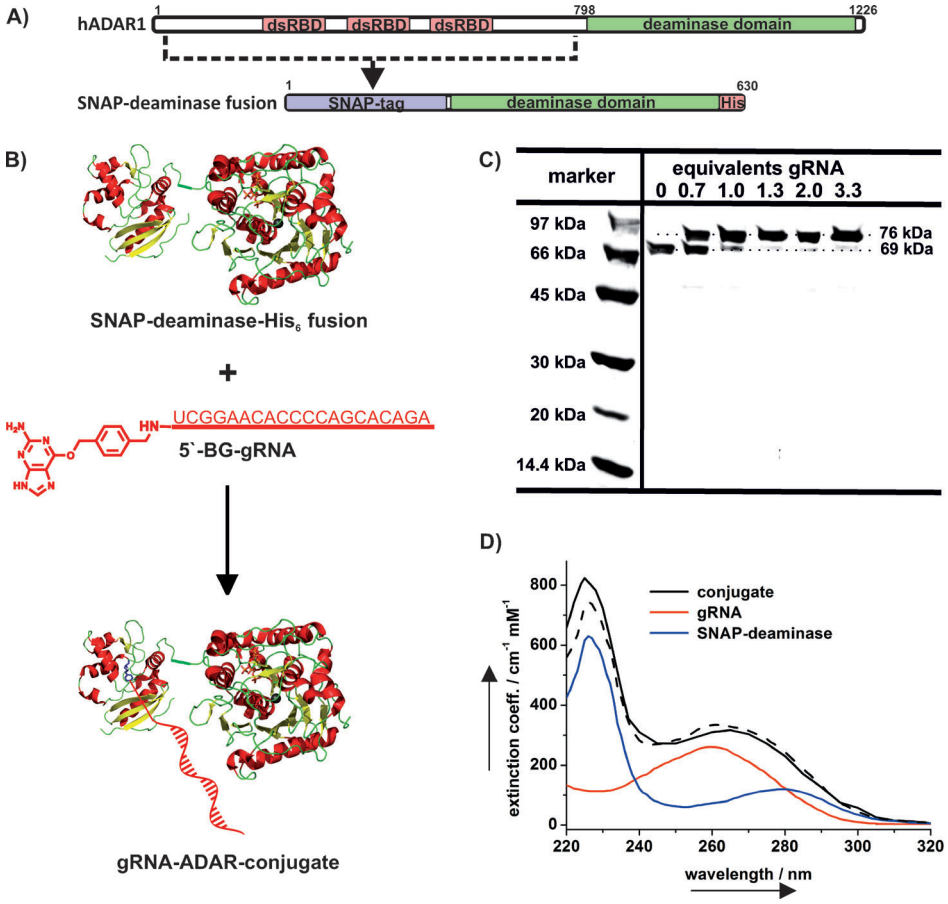

Figure 1. Converting hADAR1 into a gRNA-dependent deaminase: A) The SNAP–deaminase fusion comprises the catalytic domain of hADAR1 (aa 798–1226) lacking all natural substrate-binding domains (dsRBD), fused to the C-terminus of a SNAP-tag. B) gRNA–deaminase conjugates are formed by the reaction of BG-modified gRNA with SNAP–deaminase. C) SDS-PAGE analysis (4–20%) of the conjugation: SNAP–deaminase (1.5 μM) was incubated with various amounts of 5′-BG-modified gRNA (5′-r(UCGAUUGAACACCAUAGCAC)) for 2 h at 30°C. The samples were subjected to gel analysis without further purification. SNAP–deaminase 69 kDa; gRNA–deaminase conjugate 76 kDa. D) UV spectra of SNAP–ADAR fusion protein (blue trace), gRNA (red trace), and the conjugate (black trace). The dashed line was obtained by summing up the extinction coefficients of gRNA and SNAP–deaminase.

medium to highly transcribed mRNAs in mammalian cells (10^3–10^5 copies per cell). Usually, the conjugate was added at 2- to 10-fold excess relative to mRNA. Editing reactions were stopped by capturing the gRNA with an excess of a reverse complementary single-stranded (ss) DNA oligomer. The edited mRNA was reverse-transcribed into cDNA, amplified by PCR (Taq), and subjected to further analysis.

Figure 2 shows the typical outcome of a directed editing reaction. Sequencing of the ORF of the mRNA after reverse transcription/PCR amplification revealed the highly site-specific A-to-G conversion at the targeted UAG Stop codon with typically 60–90% conversion (Figure S2 in the Supporting Information). Notably, neither the neighboring adenosine bases nor any of the other 244 adenosine bases in the ORF are major sites for editing. However, on the basis of cDNA sequencing alone, one would easily misjudge the quality of RNA repair if overediting occurs randomly at any of the 244 nontargeted adenosine bases. Thus, the cDNA from edited mRNA was amplified (Taq), doubly restricted, ligated into an empty vector, and transformed back into competent T7express *E. coli* cells (New England Biolabs). After IPTG (isopropyl β-D-1-thiogalactopyranoside) induction, 50–75% of the colonies on the plate showed the expected cyan fluorescence, highlighting the ease by which the functionality of the nonsense mRNA transcript can be restored (Figure 2C). From such plates, 24 clones were picked and sequenced, half of which showed the positive and half the negative phenotype. As expected, all positive clones carried the reconstituted Trp^{66} codon (see the Supporting Information). Only one out of 12 clones acquired an additional but silent A-to-G mutation that may result from overediting. In accordance, within the 12 negative clones, only a single clone carried the repaired Trp^{66} codon and additional missense A-to-G mutations that impair the fluorescence and may result from overediting. Taken together, this highlights the ease by which deaminase reactivity can be harnessed to edit a single codon specifically with little overreaction.

Importantly, the editing reaction strictly relies on the covalent deaminase–gRNA assembly. Running the editing reaction in the presence of the nonmodified amino-gRNA gave no conversion over background, even though the respective double-stranded RNA motif is present (Figure 2D and Figure S3 in the Supporting Information). Obviously, removing the natural substrate-binding domains has substantially reduced the recognition of the RNA motif as a substrate,[15] and hence, the covalent attachment of the deaminase is required for RNA repair. Thus we expect the deaminase conjugate to show no or little editing on natural ADAR substrates. According to precedence from the literature,[16] cytosine was initially selected as the counter base for the editing target. Trying all four canonical bases, we found that cytosine is the best choice, followed by uridine, which still allowed substantial reactivity. The purine bases were only poor substrates, and strictly no repair was observed with guanosine (Figure S4). Thus, the natural preference of hADAR1 for cytosine as the counter base is maintained in the SNAP–deaminase conjugate.

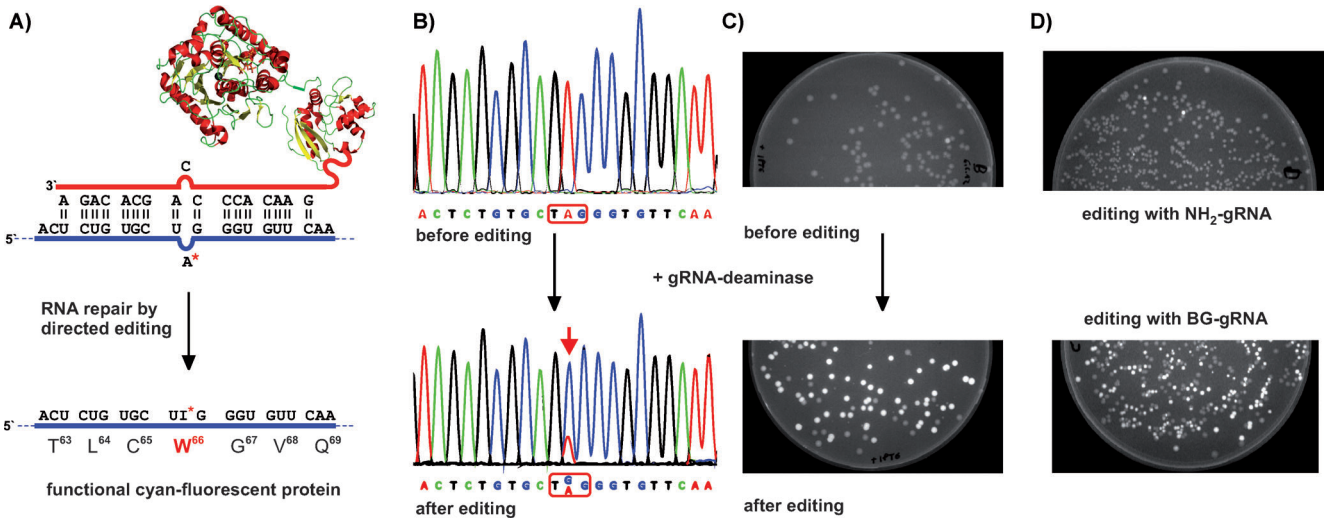

Figure 2. Repairing the Stop[66] nonsense mutation in ecfp mRNA: A) Schematic overview. B) Monitoring A-to-G conversion by sequencing after reverse transcription, [eCFP–mRNA] = 100 nM; [BG–gRNA] = 1.0 μM; [SNAP–deaminase] = 1.3 μM, 3 h. C) Plate assay: repaired genes lead to a positive fluorescence phenotype. D) Plate assay demonstrating the dependence of directed editing on conjugation: RNA repair is seen only with BG-modified gRNA and not with unmodified amino (NH_2)-gRNA.

To challenge the specificity of the gRNA-directed editing, we studied the repair of two nonfluorescent eGFP variants containing either a C65Y (UAC) or a G67S (AGC) missense mutation that is located in closest proximity to the easily editable Tyr[66] codon (UAU) (Figure 3 A and Figure S5 in the Supporting Information). Since the latter is indispensable for GFP fluorescence,[17] its overediting must be strictly avoided. However, by designing the gRNA in such a way that the sensitive Tyr[66] codon lies close to either the gRNA's 5′- or 3′-terminus, it was possible to selectively repair both missense mutations without affecting the Tyr[66] codon (Figure 3). Even though the conversion of the target bases was a little less efficient, the selectivity of the editing reactions is notable: none of the other five adenosine bases that were covered by the gRNAs are targets for overediting. The latter was also confirmed by sequencing of 20 single clones (see the Supporting Information).

Since the fluorescence spectra of eGFP and eCFP are readily distinguishable we could study the repair of one mRNA in the presence of the other. Performing the editing reaction on both mRNA substrates Y[65] eGFP and Stop[66] eCFP, but in the presence of only Y[65]-gRNA resulted in the exclusive repair of the first, indicated by the formation of green-fluorescent but not cyan-fluorescent colonies (Figure S6 in the Supporting Information). Similarly, adding only Stop[66]-gRNA gave exclusively cyan-fluorescent colonies, whereas both substrates were repaired in the presence of both gRNAs. This demonstrates again that our strategy is suitable for the highly selective repair of G-to-A point mutations even in the presence of very similar genes.

The ease by which the three codons under study (Stop, Tyr, and Ser) were repaired, the high selectivity of targeting, and the rational design suggest that our strategy for directed mRNA-editing has significant potential for medicinal applications or as a tool in basic research. For example, directed A-to-I editing could be used to shut down an enzyme's catalytic activity, a site of posttranslational modification, or a residue important for protein–protein interaction without eliminating either the targeted protein or its transcript. This is in contrast to commonly used techniques including RNA interference[18] and cre/lox recombination[19] but could be particularly advantageous for the dissection of the single functions (catalysis, signaling, binding) of proteins that are embedded in multiprotein complexes. Furthermore, single-nucleotide polymorphisms are related to various severe conditions including neural and blood diseases as well as cancer.[20] Chemically stabilized[21] guide-RNA–deaminase conjugates could potentially help to repair or to better adapt to such genetic malfunctions once the hurdle of protein transduction[22] is taken.

Experimental Section

Typically, editing reactions were performed in reverse-transcription buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 2 μM heparin, pH 8.3) at an mRNA concentraion of 50 to 100 nM in the presence of 5 to 10 equivalents of the conjugate. The conjugate was assembled in situ by adding BG-gRNA and SNAP-ADAR to the mixture with the latter in 1.3-fold excess. Mixtures were incubated for three cycles [30 °C/30 min + 37 °C/30 min] and stopped by addition of a 15-fold excess of a ssDNA oligomer reverse-complementary to the respective gRNA component. Editing was followed by reverse transcription and PCR amplification with Taq DNA polymerase. PCR transcripts were analyzed directly for A-to-G mutations by sequencing or were double-restricted (XhoI/NheI), ligated into an empty pMG211 vector, retransformed into competent T7 Express *E. coli* cells, and plated on Lysogeny broth Ampicillin/IPTG plates. The proportion of positive to negative fluorescent phenotype was

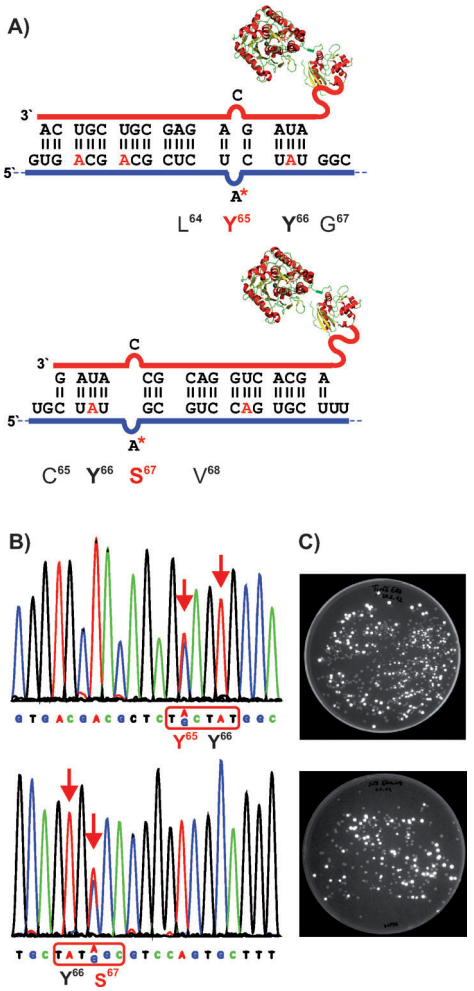

Figure 3. Challenging the selectivity of gRNA-directed editing. A) The repair of the missense mutations C65Y (UAC) and G67S (AGC) must be conducted in the presence of further A-containing codons, in particular of codon Tyr^{66} (UAU). B) Sequencing of the cDNA reveals the highly selective editing of the two target codons without the overediting of any of the five further adenosine bases. C) The plate assay confirms the repair of the mRNA by a positive fluorescence readout. For the sequencing of single colonies see the Supporting Information.

a used as a readout of the editing efficiency. Single clones were picked and sequenced to check their genotypes for unwanted overediting.

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