RNA Repair

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Improving Site-Directed RNA Editing In Vitro and in Cell Culture by Chemical Modification of the GuideRNA**

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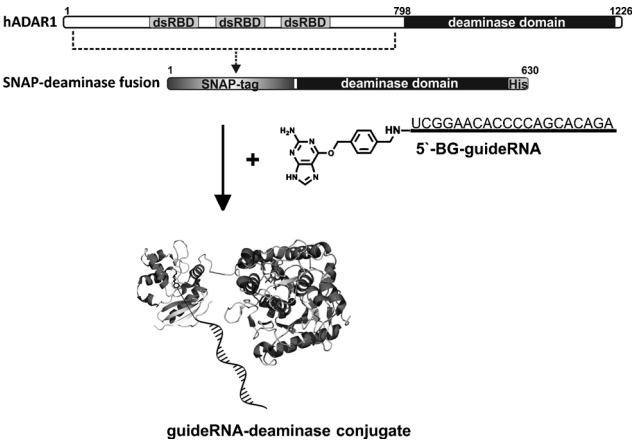
Abstract: *Adenosine-to-inosine deamination can be re-addressed to user-defined mRNAs by applying phosphothioate/2′-methoxy-modified guideRNAs. Dense chemical modification of the guideRNA clearly improves performance of the covalent conjugates inside the living cell. Furthermore, careful positioning of a few modifications controls editing selectivity in vitro and was exploited for the challenging repair of the Factor 5 Leiden missense mutation.*

RNA editing has the power to reprogram genetic information on the RNA level.[1] The outcome depends on the site at which a single adenosine-to-inosine (A-to-I) conversion occurs. If it happens in the open reading frame, the substitution of a single amino acid residue results; if it happens in an untranslated region, RNA processing is altered. Thus, directing RNA editing activity to a user-defined site of a (pre)-mRNA makes it possible to manipulate RNA and protein function with high potential for application in basic biology research and medicine (transcript repair). We recently reported a strategy for the assembly of an artificial, guideRNA-dependent RNA editing machinery that allows the application of simple Watson–Crick binding rules for the site-selective and highly rational targeting of any arbitrary codon.[2] Here, we report several improvements of the tool including a proof-of-principle for its cellular application.

To direct RNA editing, we have engineered the protein-guided human ADAR1 (adenosine deaminase acting on RNA) into a guideRNA-dependent enzyme by fusing its C-terminal catalytic domain to a SNAP-tag (Scheme 1).[2] The SNAP-tag[3] enables formation of fully defined one-to-one conjugates with guideRNAs that carry a 5′-terminal *O*6-benzylguanine (BG) modification. The RNA part of such a tool fulfills two tasks. First, it steers the deaminase domain to the target site at a user-defined mRNA, and second, it forms the secondary structure required for the highly efficient and selective editing of a single adenosine residue without overediting of nearby off-target adenosines. Most appealing is the modular nature of our approach which allows us to program the machinery to target virtually any given codon by designing a respective guideRNA.



Scheme 1. Engineering of a guideRNA-dependent deaminase. The N-terminal RNA substrate binding protein domains (dsRBD) are substituted with a SNAP-tag, which allows for the formation of defined 1-to-1 covalent conjugates with short guideRNAs 5′-terminally modified with *O*-benzylguanine (BG). The guideRNA steers editing activity towards new mRNA substrates.

Single nucleotide polymorphisms (SNP) can have a profound effect on the processing of an RNA transcript or the function of the derived protein product.[4] Hence, many SNP are directly linked to diseases. By reprogramming adenosine formally into guanosine, site-directed A-to-I RNA editing has the potential to either model, repair, or attenuate genetic disease phenotypes.[5] However, for application in research or therapy, the RNA–protein conjugate has to become effective and specific inside the cell. The direct transduction of the protein–RNA conjugate could be difficult.[6] An alternative would be the expression of the editing machinery in a genetically engineered animal or tissue culture. Whereas the SNAP-ADAR fusion is genetically encodable, the guideRNA strictly requires the chemical modification with the BG group for its proper functioning and thus is genetically not encodable.[3] Hence, with respect to in vivo application, we got interested in the stabilization of the guideRNA component by chemical modification.

Antisense oligomers are often globally modified at the ribose backbone.[7] A typical example is the antagomir[8] that contains global 2′-*O*-methyl groups and terminal phosphothioate groups in combination with a single cholesterol modification. Such modifications make the probe resistant against nucleases; thus, long-lasting microRNA knockdown of more than one week is typically obtained by a single transfection.[8] The increased lipophilicity supports their

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penetration into the cell membrane and also facilitates their trafficking between cyto- and nucleoplasm.[9] The cholesterol modification facilitates the receptor-mediated uptake in various tissues without the need for additional transfection agents.[8] Thus, the action of such probes has been reported after systemic administration to mice with remarkably low toxicity and immunogenicity.[10]

The situation is more complex for RNA probes that address a catalytically active protein complex such as the antisense strand of a siRNA after loading into the RNA-induced silencing complex (RISC). Since such probes have to fulfill their tasks inside the active site of the respective protein complex they are less receptive for modification.[11] However, it was demonstrated that chemical modification at selected sites[12] can improve pharmacokinetics, target selectivity,[13] and also toxicity and immunogenicity.[14] Various modifications including 2′-*O*-methyl, 2′-fluorine, and LNA have been reported.[11,14]

If ADAR's deaminase domain would accept chemically modified guideRNAs as substrates for editing mRNAs, an important hurdle towards in vivo application would be overcome. To not entirely block editing, we had to carefully optimize the site and degree of modification. As modifications we chose 2′-*O*-methylation and terminal phosphothioate since they are particularly simple to synthesize and commercially affordable, and have proven their utility in various applications.[7–15] To test the acceptance of 2′-*O*-methyl groups in BG-guideRNA-dependent A-to-I RNA editing, we systematically studied the effect of such modifications in our in vitro assay[2] on a nonsense Stop66 eCFP mRNA with our optimized 17 nt guideRNA. As one may expect, editing of the eCFP mRNA substrate was fully inhibited by a protein–RNA conjugate containing a guideRNA that carried three 2′-methoxy groups centered at the counter base of the targeted adenosine (Figure 1 B versus 1 A). It was shown before that densely modified nucleic analogues inhibit RNA editing.[16] However, modifying only the counter base of the targeted adenosine with a single 2′-methoxy group gave not complete but rather roughly 50 % inhibition (Figure 1 C). Notably, a single modification one nucleotide up- or downstream from the counter base had nearly no or only very little influence on the overall editing yield (Figure 1 D,E). Thus, full inhibition can only be achieved by cooperative action of several proximate 2′-modifications. We then tested a guideRNA that was densely modified with 2′-methoxy groups leaving only a little gap of three natural ribonucleotides centered around the counter base, and that further carried two phosphothioate modifications at the 5′-terminus and four at the 3′-terminus, as antagomirs[8] typically do. Even though this 17 nt duplex was heavily chemically modified on the guide strand, a very good editing yield of ≥ 80 % was obtained with SNAP-ADAR1 (Figure 1 F). Thus, antagomir-like guideRNAs are well accepted by the deaminase domain of hADAR1 if they contain a minimal gap (3 nt) of unmodified RNA at the target site. Nucleic acid dependent enzymes typically tolerate less extensive chemical modification on the guide. For instance, gapmers that redirect RNaseH activity require a gap of 7 to 10 unmodified deoxyribonucloetides,[17] whereas the antisense strand of siRNAs can tolerate 2′-fluorine,[12] but is disabled in the presence of global 2′-methoxylation.[7,8,11] Thus applying a very limited number of modified bases is usually recommended.[11] Our finding was unexpected but is highly important: in the future it may allow us to express SNAP-ADAR fusions inside an animal or a tissue and to then manipulate protein or RNA function under conditional control by administration of a chemically stabilized guideRNA.

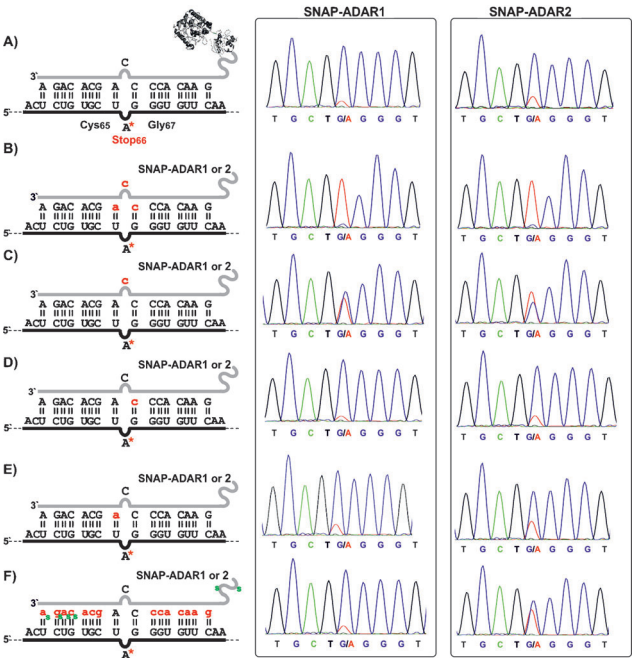

Figure 1. Effect of chemically modified guideRNAs on editing of the Stop66 codon in eCFP mRNA (mRNA black; guideRNA gray; the targeted adenosine is marked with an asterisk; 2′-OMe-modified bases are indicated by small red letters, phosphothioate linkages by green "s"). Editing yields are estimated from the areas for adenosine (red) versus guanosine (blue) in the respective sequencing traces with SNAP-ADAR1 (left) and SNAP-ADAR2 (right). Editing conditions: 3 h at 30/37 °C; 50 nM mRNA, 500 nM BG-guideRNA, 650 nM SNAP-ADAR1 or -ADAR2, 75 mM KCl, 25 mM Tris-HCl, 10 mM DTT, 0.75 mM $MgCl_2$, 2 μM heparin, pH 8.3). One full sequencing trace is given in the Supporting Information (Figure S3).

To further illustrate that approach we transiently expressed $SNAP_f$-ADAR1 and the fluorogenic reporter gene under control of a CMV promotor in HEK 293T cells and stimulated transcript repair by transfection of the guideRNA. For this, $SNAP_f$-ADAR1 and Stop66 eCFP were subcloned into the pcDNA3.1 vector. Wild-type (Trp66) eCFP served as a positive control. Cotransfection of both plasmids in a 1-to-1 ratio was achieved with lipofectamine 2000 in a 24-well format. After 24 h, transfected cells were detached with trypsin, distributed evenly over several wells in a 96-well plate, and incubated for 24 h prior to lipofection with various guideRNAs (50 pMol in 150 μL/well). After one day of incubation, the fluorescence phenotype was analyzed by microscopy.

Whereas the positive control (Figure 2 A) gave a clearly visible fluorescence signal, no fluorescence was observed when the nonfunctional Stop66 eCFP was transfected in the

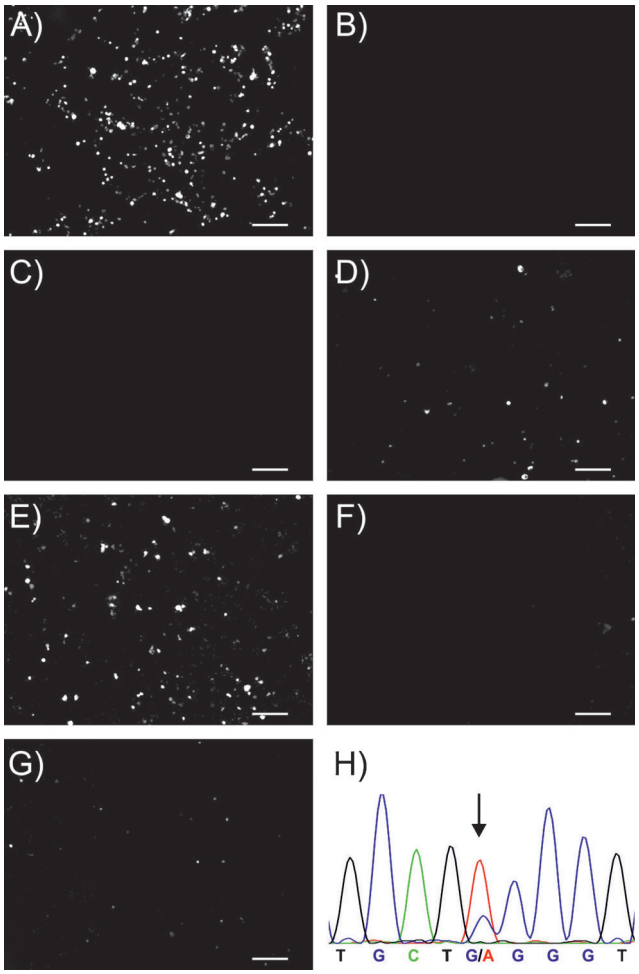

Figure 2. Directed RNA editing in 293T cells. Cells were cotransfected with equal amounts of reporter gene (Stop66 eCFP or wild-type) and $SNAP_f$-ADAR1 (or empty pcDNA3.1). After 24 h guideRNAs were transfected and 24 h later the fluorescence phenotype was analyzed by fluorescence microscopy. A) Positive control (wild-type eCFP with $SNAP_f$-ADAR1 and BG-antagomir-guideRNA); B) negative control 1 (Stop66 eCFP/empty pcDNA3.1/BG-antagomir-guideRNA); C) negative control 2 (Stop66 eCFP/$SNAP_f$-ADAR1/no guideRNA); D)–G) experiments with Stop66 eCFP/$SNAP_f$-ADAR1 and various guideRNAs: D) standard BG-guideRNA; E) BG-antagomir-guideRNA; F) standard NH_2-guideRNA; G) NH_2-antagomir-guideRNA. The scale bar represents 200 μm. H) Sanger sequencing of the RNA extracted from cells treated as in experiment (E). For details on cell culturing see the Supporting Information.

presence of either $SNAP_f$-ADAR1 or the BG-modified antagomir-like guideRNA alone (Figure 2B,C). For further controls see Figure S6. In contrast, CFP fluorescence was detectable when Stop66 eCFP was coexpressed with $SNAP_f$-ADAR1 and subsequently transfected with our standard BG-modified guideRNA lacking 2′-methoxy and phosphothioate modifications (Figure 2D). However, compared to the positive control, the number of bright fluorescing cells was low. In order to estimate the effect of further chemical modification we transfected Stop66 eCFP/$SNAP_f$-ADAR1-coexpressing cells with the BG-modified antagomir-like guideRNA. Compared to the standard guideRNA (Figure 2D), the chemically stabilized guideRNA displayed a markedly increased number of fluorescent cells (Figure 2E), hence clearly demonstrating the expected beneficial effects of chemical modification in a cellular environment.

In vitro, the editing reaction benefits strongly from the covalent conjugation between guideRNA and deaminase. Applying 5′-amino (NH_2)-modified guideRNAs instead of the 5′-BG-modified guideRNAs typically results in a reduced or even abolished editing activity depending on the in vitro editing conditions.[2] Thus we tested the cellular transcript repair in the presence of the corresponding NH_2-guideRNAs. As expected, no (Figure 2F), or only very little CFP fluorescence (Figure 2G) was restored by applying guideRNAs that cannot address the deaminase domain by means of covalent attachment. Apparently covalent conjugation is fast enough inside the cell and is required for efficient transcript repair. The conjugation kinetics of our applied $SNAP_f$-tag variant for BG-tagged moieties was reported to be $2.8 \times 10^4\,M^{-1}s^{-1}$ and was previously shown to be sufficient for fluorophore conjugation in the cytosol of living mammalian cells.[18]

To clearly demonstrate that the fluorescence phenotype was due to RNA editing at the targeted Stop66 codon, we extracted the total RNA from the cells, removed possible DNA contaminations with DNaseI, reversely transcribed the eCFP mRNA with a specific backward primer, and amplified the cDNA through Taq-PCR with CFP-specific primers. In agreement with the fluorescence imaging, Sanger sequencing revealed the highest editing yield for the antagomir-like BG-guideRNA (Figure 2H) and no detectable editing for the control experiment expressing $SNAP_f$-ADAR1 but lacking the guideRNA (Figure S7A). Natural editing enzymes achieve selective and up to quantitative editing, similar to our optimized in vitro directed editing approach. Since many parameters remain to be optimized for the cellular assay, including the length, sequence, concentration, modification, and transfection of the guideRNA, as well as the ratio and transfection of the reporter and SNAP-ADAR genes, we expect further improvements in the future.

As a last control, we transfected Stop66 eCFP/$SNAP_f$-ADAR1 co-expressing cells with a 2′-methoxy-phosphothioate modified BG-guideRNA that binds around Trp codon 58, thus 24 nt upstream of the targeted codon. Even though this BG-guideRNA is capable of repairing a Stop58 eGFP mutation there was no detectable repair of the Stop66 eCFP mutation (Figures S6H and S7F). This is in very accordance with our experimental experience. Re-directed RNA editing is highly selective and requires the positioning of the targeted adenosine in a well-defined secondary structure.[2]

Chemical modification of the guideRNA may also improve substrate specificity in a cellular application, because random binding of the probe to a partially complementary sequence in the transcriptome may less often lead to unwanted off-site editing.[15] Furthermore, chemical modification may not only improve the pharmacological properties including lifetime and off-site effects, but could also improve the editing selectivity. We demonstrate this for the repair of the Factor 5 Leiden polymorphism. This disease-causing single point mutation ($G^{1746} \rightarrow A$) represents the most abun-

dant genetic risk factor in heritable multifactorial thrombophilia in the Caucasian population.[19] Due to the point mutation, a single amino acid substitution (R534→Q) appears at the Protein C dependent proteolytic cleavage site (R533R534) of the blood coagulation factor F5. Whereas the heterozygous defect is accompanied by an only minor increase in thrombosis risk (ca. 8-fold), the homozygous defect has a much more pronounced effect (>80-fold increased risk).[19] Directed RNA editing has the potential to compensate for this genetic defect by its repair at the RNA level. However, a look into the gene revealed a very adenosine-rich and thus highly challenging target site (Figure 3A). The glutamine codon (5′-C*A*A) is known to be more difficult to activate than the amber stop codon.[20, 2b]

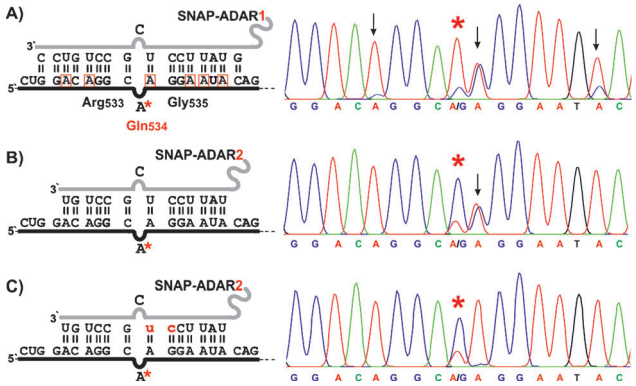

Figure 3. Repair of the F5 Leiden polymorphism. Potential off-target adenosines are highlighted with red boxes. Overedited sites are marked with black arrows, the targeted site with an asterisk. Editing conditions: 50 nM R534Q F5 mRNA, 200 nM BG-guideRNA, 350 nM SNAP-ADAR1/2; 3 h at 30/37 °C in 75 mM KCl, 25 mM Tris-HCl, 10 mM DTT, 0.70 mM $MgCl_2$, pH 8.3. An overview of the editing results for all guideRNAs with SNAP-ADAR1 and SNAP-ADAR2 is shown in the Supporting Information together with one complete sequencing trace (Figures S4 and S5).

The repair reaction was studied on a 1000 nt long piece of the F5 transcript containing the F5 Leiden mutation centrally. This was required since both the F5 pre-mRNA (>70 kb) and the mature mRNA (7 kb) are too large[19d] for our standard editing assay.[2] We started the repair study with a 17 nt long guideRNA that puts the targeted adenosine into an A/C mismatch in the middle of the guideRNA/mRNA helix. Even though none of the 330 adenosines outside of the helix have been edited, we found massive overediting at neighboring off-target adenosines inside the guideRNA/mRNA duplex (Figure 3A). Specifically, editing was observed at four sites, with the targeted site being barely activated (≤20% yield). The highest editing yield (ca. 50%) was obtained at the direct neighbor of the targeted base. Since ADAR2 is known to edit the Q/R site in the glutamate receptor with up to quantitative yield,[1] we made use of our modular approach and changed the deaminase domain in our SNAP fusion protein from that of hADAR1 to that of hADAR2 (see the Supporting Information). Indeed, this helped to activate the 5′-C*A*A codon considerably (ca. 70% yield, Figure 3B). Shortening the guideRNA (from 17 to 14 nt) was sufficient to suppress overediting at the distal sites; however, the strong overediting (ca. 50%) at the adenosine directly neighboring the targeted base stayed unaffected (Figure 3B). Our work on 2′-*O*-methylated guideRNAs (Figure 1A–G) suggested that incorporation of 2′-methoxy groups around the off-targeted adenosine may provide a means to suppress overediting in this very delicate codon context. To avoid affecting the targeted adenosine, we placed only two modifications on the guideRNA, one opposite the off-site adenosine and one opposite its neighboring guanosine. Indeed, overediting was completely abolished without affecting editing at the target site for which the overall yield remained roughly 70% (Figure 3C). Such repair yields would be more than sufficient to attenuate the disease phenotype.[19] Thus chemical modification of the guideRNA is not only useful to increase nuclease resistance but provides a means to finetune editing selectivity.

In summary, we have demonstrated the strength and applicability of site-directed RNA editing as a rational approach for RNA repair. Chemical modification turned out to be a reliable means to suppress overreaction and steer selectivity. Importantly, we found that even massive chemical modification including global 2′-methoxy and terminal phosphothioate groups is well accepted as long as a small gap of three nonmodified ribonucleotides is maintained. Furthermore, we have demonstrated the functioning of the tool inside the cell. To our knowledge this is the first example of the assembly of an enzymatically active, covalent protein nucleic acid (analogue) conjugate inside the living cell and may represent an attractive strategy for the spatially or temporally controlled assembly of protein arrays in general.[21] In contrast to competing strategies that apply the protein–RNA recognition by means of the MS2 or λN phage system for assembly,[22,21] our guideRNAs are particularly short (20 nt) and lack additional BoxB RNA hairpin (19 nt) and linker (10–20 nt) motifs required for recognition and spacing that make other guideRNAs ≥60 nt long.[22] Our stabilized 20 nt short guideRNAs are particularly suitable for transfection and are supposed to be too small to elicit an immune response.[7–15] The necessity to transfect our guideRNAs instead of expressing them encourages us to think about introducing further chemical modifications that endow our probes with additional layers of control, such as photoactivation. Our findings dramatically improve the prospect of directed RNA editing for in vivo applications in basic biology research and medicine. Along those lines, we demonstrated the first repair of a disease-causing missense point mutation in vitro. This required the exchange of the deaminase domain of the SNAP fusion protein from hADAR1 to that of hADAR2 and the careful chemical modification of the guideRNA to target the codon in its highly challenging adenosine-rich sequence context. The high modularity of the SNAP-deaminases in combination with their useful tolerance for chemical modification highlight the potential of this approach for site-directed RNA repair.

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