Switching Protein Localization by Site-Directed RNA Editing under Control of Light

Paul Vogel, Alfred Hanswillemenke, and Thorsten Stafforst*

University of Tübingen, Interfaculty Institute of Biochemistry, Auf der Morgenstelle 15, 72076 Tübingen, Germany

Supporting Information

ABSTRACT: Site directed RNA editing is an engineered tool for the posttranscriptional manipulation of RNA and proteins. Here, we demonstrate the inclusion of additional N- and C-terminal protein domains in an RNA editing-dependent manner to switch between protein isoforms in mammalian cell culture. By utilization of localization signals, a switch of the subcellular protein localization was achieved. This included the shift from the cytoplasm to the outer-membrane, which typically is inaccessible at the protein-level. Furthermore, the strategy allows to implement photocaging to achieve spatiotemporal control of isoform switching. The strategy does not require substantial genetic engineering, and might well complement current optogenetic and optochemical approaches.

KEYWORDS: RNA editing, photocontrol, photocaging, protein localization, optogenetics, epitranscriptomics, gene regulation

During expression genetic information is diversified by various mechanisms. Even when encoded in a single genetic locus, many proteins occur in several isoforms, which result from alternative promoter usage or alternative splicing. Another way of diversification is a process called RNA editing.1,2 This refers to the insertion or deletion of nucleotides and to the enzymatic deamination of cytosine and adenosine resulting in the formation of uridine and inosine, respectively. Upon editing in the open reading frame (ORF) single amino acids can be recoded. Furthermore, RNA editing can interfere with RNA splicing, microRNA activity, and RNA stability. Such diversifications can affect almost any property of a protein including substrate specificity, catalytic efficiency, protein localization, stability, and others. As correct subcellular localization is essential for proper functioning, mislocalization can act as a strategy to control a protein’s function. One example is the cytosolic sequestering of transcription factors like NF-κB or the glucocorticoid receptors, which translocate to the nucleus in response to specific signaling cues. Synthetic biology has exploited the induction of translocation as a strategy to control genetic networks. One example for the latter is the engineered Cre-ER(T2) system for the conditional switch of gene function in vivo.1,4

The information about the subcellular localization is typically encoded in short peptide-segments, so-called localization signals.5 Several signals, like the nuclear localization signal (NLS), can be found anywhere in a protein. Whereas others, like the ER-targeting sequence, are typically found in the N-terminus and are proteolytically cleaved off during translation.6 If protein isoforms differ in their localization, such signal peptides are typically in- or excluded by alternative promoter or splice sites usage. We were wondering if site-directed RNA editing could be harnessed for that purpose.7

We and others have recently engineered artificial guideRNA-dependent editing machines that allow for the introduction of single A-to-I substitutions at targeted sites in selected transcripts inside living cells, a process called site-directed RNA editing.8 To achieve this, we have fused the catalytic domain of a human adenosine deaminase acting on RNA (hADAR) with a SNAP-tag that allows for the formation of highly defined 1-to-1 covalent conjugates between the guideRNA and the deaminase.9 The approximately 20 nt long guideRNA steers the deaminase to any arbitrary transcript in a readily programmable way. As the deaminase acts only on double-stranded RNA the guideRNA component provides the basis for substrate specificity. By chemical modification and sequence refinement, the selectivity and efficiency of the editing reaction can be further fine-tuned.10,11 So far, we and others have applied site-directed RNA editing strategies in human cell culture,10 in living organisms,12 and in Xenopus eggs,9 to manipulate reporter genes and to repair disease-related mutations in CFTR9 and PINK113 mRNAs. Furthermore, we recently demonstrated the possibility of controlling the guideRNA—deaminase assembly by light, which enabled us to extent RNA editing by photocontrol in vivo.12

Here, we now demonstrate a simple strategy to apply RNA editing for triggering the inclusion of an additional peptide signal into both, the N- or the C-terminus of a protein. We apply the strategy for the inclusion of a nuclear localization signal (N- or C-terminal) and for the switching between a
RESULTS AND DISCUSSION

General Considerations. The C-terminal inclusion of an additional peptide signal appears particularly straightforward by putting the signal into the 3′-UTR directly behind an amber Stop codon (UAG), Scheme 1. Upon editing the Stop codon to Trp (UIG) the additional signal is inserted C-terminally. The analogous strategy at the N-terminus appears more challenging. We explore here the activation of an alternative Start codon in the 5′-UTR, as it is conceivable to edit an isoleucine codon (AUA) into a methionine/Start codon (AUI), Scheme 1. Prior to editing, the downstream Start codon would be used only. However, after editing the upstream Start should dominate, as cap-dependent translation typically applies the first Start codon after the cap. Nevertheless, site-directed RNA editing inside the 5′-UTR has not yet been reported. Also within natural editing sites, editing in the 5′-UTR is strongly underrepresented. Thus, it was unclear if the preinitiation complex of translation and the editing machinery will interfere. To assess both strategies in a comparable manner, we decided to start with the inclusion of a nuclear localization signal (NLS) derived from the SV 40 Large T-antigen (PKKKRKV), which can be put to both, the N- or C-terminus. To visualize the localization phenotype, we chose the transcript of the editing enzyme (SNAP-ADAR2) as the editing target. On one hand, the enzyme is strictly localized in the cytoplasm when lacking an NLS. On the other hand, the enzyme is readily stained with fluoresceine-O6-benzylguanine (BG-FITC) to assess its localization by fluorescence microscopy. Furthermore, this procedure allowed us to stay with the ectopic expression of a single construct which simplified transfection and phenotypic analysis.

Editing-Dependent Inclusion of the NLS into the C-Terminus under Transient Expression. According to Scheme 1, two plasmids were constructed that contain SNAP-ADAR2 under control of the CMV promotor. In one construct, the NLS was put in frame at the C-terminus (SA-TGG-NLS). When transfected into 293T cells and BG-FITC-stained 48 h later, a clear nuclear localization was visible (Figure 1a). The other construct contained a single G-to-A mutation between the SNAP-ADAR and the NLS which inserts a premature Stop codon and thus shortens the open reading frame (SA-TAG-NLS). When expressed and stained comparably, a clear cytoplasmic phenotype was visible. The latter construct was the substrate to study the editing-dependent phenotype switch.

For editing, 293T cells were first transfected with SA-TAG-NLS (or SA-TGG-NLS in the control) and were then reverse transfected with a guideRNA. When the matching guideRNA was used, BG-FITC staining revealed a clear appearance of nuclear SNAP-ADAR2 protein (Figure 1a) that resembles the phenotype of the positive control. We found this new, mixed cyto-/nucleoplasmic phenotype in 48 ± 9% of the transfected cells. Sanger sequencing revealed an editing yield of 74 ± 9%. We assume two reasons for the mixed (cytoplasmic/nuclear) phenotype after editing. First, editing was incomplete, and second, some of the stained SNAP-ADAR2 protein was old protein from the SNAP-ADAR expression prior to induction of the editing event by transfecting the guideRNA. The isoform

"The NLS has been included either N- or C-terminally into the SNAP-deaminase protein. The Igk-leader sequence, which signals plasma membrane localization, has been included N-terminally into an HA-tagged eGFP. The C-terminal platelet-derived growth factor receptor transmembrane domain (PDGFR-TMD) is a single transmembrane α-helix that anchors the protein to the plasma membrane by pointing the N-terminus outside. The expected localization phenotype (cytoplasm, nucleoplasm or outer membrane) is indicated."
switch was strongly dependent on editing. It did neither occur in the presence of an NH₂-guideRNA incapable of conjugation,12 nor in the presence of a BG-guideRNA with a mismatching (mm) sequence (Figure 1a). However, due to the high levels of SNAP-ADAR2 protein and its transcript under transient expression, low levels of guideRNA-independent editing were detectable (Figure 1a, graph). Even though this low-level editing did not result in a visible nuclear localization phenotype, we aimed to further improve the performance of the system by genomic integration of the SNAP-ADAR construct.

C-Terminal NLS-Inclusion Works Also under Genomic Expression. To obtain a weaker and more homogeneous expression, the respective constructs were integrated as a single copy into the genome of 293 Flip-In cells under control of the Tet-on CMV promotor (inducible genomic expression). Fluorescence microscopy confirmed the homogeneous, inducible and much weaker expression of the editase under genomic control (Figure 1b). Even though this low-level editing did not result in a visible nuclear localization phenotype, we aimed to further improve the performance of the system by genomic integration of the SNAP-ADAR construct.

Editing-Dependent Inclusion of the NLS into the N-Terminus (Transient Expression). As depicted in Scheme 1, two plasmids were constructed that contain two Start codons each embedded in a strong Kozak sequence (5'-CCACC-AUG-3').19 One of the Start codons was located in front and one behind the NLS. In the construct ATGG-NLS-SA, both Start codons are appropriate to start translation. According to the scanning model of cap-dependent translation one expects this construct to predominantly use the Start codon prior to the NLS and thus to express the full NLS-SNAP-ADAR2 protein.14−16 Accordingly, transient expression of this construct in 293T cells showed exclusive nuclear localization of SNAP-ADAR (Figure 2a). The construct ATAG-NLS-SA differs from the latter by a single G-to-A mutation in the upstream Start

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Editing-dependent switch from SNAP-ADAR2 to SNAP-ADAR2-NLS under transient (a) and genomic (b) expression. (a) Fluorescence imaging of FITC-stained SNAP-ADAR (green) and Hoechst 33342-stained nuclei (blue). SA-TGG-NLS is the positive control for the nuclear localization phenotype after editing. Quantitative analysis of the editing experiment: Blue shows the editing yield from Sanger sequencing. Red shows the amount of cells that are positive for SNAP-ADAR expression and show nuclear localization. mm BG-gRNA: mismatching BG-guideRNA. Black bars show the standard deviation from N = 3 independent experiments. The scale bars represent 20 μm. (b) Analogous experiment as in panel (a), but under genomic expression of the SNAP-ADAR constructs. n.d. = neither RNA editing nor nuclear localization was detectable. Further data and controls are shown in the Supporting Information, Figure S1−S3 for transient and S4−S6 for genomic expression.
codon, thereby creating a 5′-CCACCAUA*G sequence that is supposed to be inappropriate to start translation prior to editing (AU*A) but to turn into a strong initiation signal after editing (AU*I). Transient expression of this construct gave almost exclusive cytoplasmic localization of SNAP-ADAR. Only a small number of cells (10 ± 4%) showed a faint nuclear staining (Figure S10), which might result from a minor translation initiation from the unedited AUA Start codon, as it is embedded in a very strong sequence context. However, in a similar setting it was reported that the plasmid-borne sequence 5′-CCACCAG is unable to initiate translation when transfected into COS cells.20 Clearly, the faint nuclear staining was not due to (guideRNA-independent) editing, as the editing yield in absence of the guideRNA was below the detection limit (≤2%). For editing, 293T cells were transfected with either of the two constructs and reverse transfected with a guideRNA. Protein localization was analyzed by fluorescence microscopy after BG-FITC staining. After transfection of the editing substrate (ATAG-NLS-SA) and the matching BG-guideRNA, we found a clearly visible nuclear staining that resembled that of the positive editing control (ATGG-NLS-SA), Figure 2a. Similar to the results at the 3′-UTR, we found a mixed nucleo-/cytoplasmic phenotype in 57 ± 5% of all cells. After editing the nuclear staining of the protein was much stronger compared to the occasional faint nuclear staining observed prior to editing (Figure S10). Sequencing of the mRNA revealed an editing yield of 58 ± 4%, in good agreement with the mixed phenotype. Again, the isoform switch was dependent on the editing event and did not happen in the presence of a mismatching or conjugation-incompetent NH2-guideRNA.

**Editing in the 5′-UTR under Genomic Expression Requires an Activated Deaminase.** Again, we tested editing under genomic expression of the 5′-UTR constructs. Upon induction, both constructs behaved as expected showing either the strict nuclear (ATGG-NLS-SA) or cytoplasmic localization (ATAG-NLS-SA) with strongly reduced but homogeneous expression over the entire culture. Compared to the expression under transient conditions, the occasional appearance of faint nuclear staining in the ATAG construct was almost abolished (below 3%). However, the editing-dependent isoform switch was disappointing. The nuclear phenotype was visible in no more than 11 ± 4% of the cells. However, this matched the low editing levels of 13 ± 1% (Figure 2b). The editing reaction might suffer from the comparably low concentration of editase and substrate, which might slow down the editing reaction. To test if a faster enzyme would help to improve the performance, we engineered two new cell lines that contain again either the ATAG or ATGG construct, but now with a SNAP-ADAR2* protein that contains a well-described, single point mutation in the deaminase domain (E488Q) that is reported to speed up deamination by at least 1 order of magnitude.21

![Figure 2. Editing-dependent switch of SNAP-ADAR2 to NLS-SNAP-ADAR2 under transient (a) and genomic (b) expression. Experiment and analysis follows the description given in Figure 1. SA* marks the construct with the activated E488Q deaminase. The scale bars represent 20 μm. Further data and controls are shown in Figures S7–S9 for transient and S11–S13 for genomic expression.](image-url)
 Isoform Switching Can Be Controlled by Light. Light is an attractive trigger to manipulate biological systems.17 We tested if a recently introduced strategy to control the editing process by controlling the assembly reaction could be applied for the light-control of 5′-UTR editing. As described earlier, guide RNAs have been made that mask the SNAP-reactive BG moiety with the Npom photocage to render it inactive for the guide RNAs. A conceivable photocaged amino acids.24 The latter strategies are feasible because trafficking into the nucleus is a posttranslational mechanism applied to fully folded proteins. As RNA editing happens before translation, isoform switches become feasible that are decided cotranslationally and thus are impossible to control at the protein level. A conceivable

ISOFORM SWITCHING CAN BE CONTROLLED BY LIGHT

Light is an attractive trigger to manipulate biological systems.17 We tested if a recently introduced strategy to control the editing process by controlling the assembly reaction could be applied for the light-control of 5′-UTR editing. As described earlier, guide RNAs have been made that mask the SNAP-reactive BG moiety with the Npom photocage to render it inactive for the assembly reaction.22 Then, the editing reaction can be started by treating the cells under the microscope with a short UV-light pulse (365 nm, 5 s). First, we studied the system with the wildtype ATAG-NLS-SA construct under transient expression. The light flash had no effect on the negative (NH2-guideRNA) or the positive editing control (BG-guideRNA) in terms of editing yield (4 ± 2% and 53 ± 1%) and localization phenotype (19 ± 4% and 57 ± 5%), Figure 3a. However, when applying the Npom-protected BG-guideRNA, a clear photoinduction of editing yield and isoform switch was detectable. Without irradiation, 19 ± 6% of cells showed a faint nuclear staining, whereas 53 ± 12% of the cells showed the switch to a clear nuclear staining after irradiation (Figure 3a). This was in accordance with the photoinduced change of editing levels from 13 ± 5% before to 40 ± 8% after irradiation. As before, the ATAG-NLS-SA construct suffered from the occasional formation of faint nuclear staining under transient expression.

Thus, we also tested photocontrol under genomic expression. As before, editing yields and phenotype switching were dissatisfying with wildtype enzyme and stayed below 20% (Figure 3b, graph). However, the E488Q variant of the deaminase was helpful again, and the positive editing control (BG-guideRNA) gave robust nuclear staining in 46 ± 4% of the cells, matching the respective editing yields of 45 ± 4% (Figure 3b). The negative editing control (NH2-guideRNA) showed virtually no editing (≤4%) and also the occasional faint nuclear staining was strongly reduced (≤4%). When applying the Npom-protected guideRNA, a clear photoactivation was visible. Before irradiation 12 ± 2% of the cells showed the nuclear staining, whereas 41 ± 3% showed nuclear staining after irradiation. Again the effect was clearly depending on the editing yields which changed from 13 ± 2% before to 37 ± 5% after irradiation, see Figure 3b. Thus, protein isoforms can be switched by light simply by photocontrolling the assembly reaction of editase and guideRNA, both under transient and genomic expression.

5′-UTR Editing Enables to Switch Localization from the Cytoplasm to the Outer Membrane. Induction of protein translocation from the cytoplasm to the nucleoplasm under control of small molecules and/or light has been achieved earlier, either by engineering fusion proteins to become controllable by small molecules (i.e., the Cre-ER(T2) system)23 or by the ectopic expression of proteins with site-specifically photocaged amino acids.24–26 The latter strategies are feasible because trafficking into the nucleus is a posttranslational mechanism applied to fully folded proteins. As RNA editing happens before translation, isoform switches become feasible that are decided cotranslationally and thus are impossible to control at the protein level. A conceivable

The two new constructs behaved indistinguishable from their less active counterparts in terms of expression level, homogeneity and localization phenotype. However, in the editing experiment the new constructs showed a robust isoform switch. A clear change to the mixed nuclear/cytoplasmic phenotype was found in 46 ± 4% of the cells, resulting from an improved editing yield of 42 ± 2% (Figure 3b). As seen before, the switch was fully dependent on editing. Consequently, the nuclear phenotype was seen in ≤4%, when no guideRNA, a mismatching guideRNA, or an NH2-guideRNA was used, reflecting the low editing yields obtained under these conditions (≤4%). Substitution of the deaminase by a more active variant boosted the performance of the system by 3-fold in terms of editing yields and number of cells with a phenotypic switch and brought the editing at the 5′-UTR to a level comparable to that at the 3′-UTR.

Figure 3. Photoinduced switch of SNAP-ADAR2 to NLS-SNAP-ADAR2 under transient (a) and genomic (b) expression. SAfl marks the construct with the activated E488Q deaminase. The scale bars represent 20 μm. Further data is shown in Figures S7–S9 for transient and Figures S11–S16 for genomic expression.
example is plasma membrane localization. The respective signal peptides are found in the very N-terminus of a protein. Once the nascent signal peptide leaves the exit at the ribosome, it is recognized by the signal recognition particle that recruits the translating ribosome to the ER. At the ER, translation continues, the signal peptide is cleaved off during translation inside the ER and the protein is inserted into the membrane cotranslationally.

We explored how RNA editing can be used to switch protein isoforms from a cytoplasmic to a membrane-anchored localization. For this a construct was made that contains an editable Start codon (AUA*) followed by the 22 amino acid Igκ chain leader sequence, an alternative Start codon (AUG), and an HA-tagged GFP protein (Scheme 1). At the very C-terminus, the construct contains the transmembrane domain (TMD) of the PDGF receptor that anchors the protein to the plasma membrane displaying the GFP and the HA-tag to the extracellular side of the cell. The analogous ATGG construct served as the positive editing control. To assess the phenotype, immunofluorescence microscopy was applied.

Under transient expression (293T cells) of the positive control (ATGG), the HA-GFP is clearly localized to the outer membrane, as visualized by a rim-like anti-HA-immunostaining in fixed but not permeabilized cells (Figure 4a). In contrast, the negative editing control (ATAG) gave no rim-like anti-HA-staining (Figure 4b). However, when cells were permeabilized prior to immunostaining (Figure S21), the cytoplasmic expression of the construct was clearly detectable. When cotransfecting the ATAG construct with SNAP-ADAR2-BFP and reverse transfecting the matching BG-guideRNA, the HA-immunofluorescence showed again the rim-like staining of the outer membrane in 43 ± 2% of the cells that have been positive for GFP and BFP fluorescence (Figure 4c). This phenotypic switch was again clearly depending on the editing yield (64 ± 5%). It did not occur in the absence of a guideRNA or in the presence of a mismatching or NH₂-guideRNA (Figure 4b).

Translocation to the Outer Membrane Can Be Controlled by Light. Finally, we tested to switch the isoforms under control of light. As before, we put the Npom photocage on the guideRNA. When applying the Npom-BG-guideRNA, a modest residual editing activity was detected (9 ± 1%), however, no outer-membrane staining was detectable (<2%, Figure 4d). After irradiation with 365 nm light a clear membrane staining became visible in 31 ± 3% of the cotransfected cells (Figure 4e). Accordingly, the editing yield increased from 9 ± 1% prior to 44 ± 5 after irradiation. UV-irradiation had no influence on the editing yield or localization phenotypes of the positive (BG-guideRNA) or negative (NH₂-guideRNA) editing controls (Figures S17–S20). Overall, isoform switch from cytoplasmic to the outer membrane can be controlled at the posttranscriptional level, and photocontrol is readily included.

■ CONCLUSION AND OUTLOOK

RNA editing can be applied to switch protein isoforms. This is not restricted to the recoding of amino acids or splice sites, but can be harnessed for the inclusion of additional N- or C-terminal peptide signals by editing of Start and Stop codons. UTRs in mammals are typically around 100 nt long, but can extent to 1000 nt or longer. Thus, even the N- or C-terminal inclusion of large protein domains is conceivable. Our artificial editing strategy that relies on the RNA-guided SNAP-tagged deaminases enables this without detectable interference with translation and translation initiation. It can be accomplished either under transient or genomic expression. The usage of the SNAP-deaminases further allows for a ready inclusion of light-control. The method might well complement current methods in synthetic biology, including optogenetics and other optochemical approaches. On one hand it enables light-controlled isoform switches that are impossible at the protein-level. This holds particularly true for phenotypes that separate already during translation and thus are inaccessible.
with caged or otherwise engineered proteins, as demonstrated by the switch to an outer-membrane anchored isoform. On the other hand, the method might complement approaches that depend on the light-dependent (in)activation of genes, which typically require massive genetic engineering. To our knowledge, this is the first report about redirecting protein localization from the cytoplasm to the membrane. In combination with light-control, our tool could provide new opportunities to address biological questions in basic research. In the future, proteins might be steered to the cell surface by using light-activated RNA editing to manipulate intracellular signaling but also extracellular events like cell–cell and cell–matrix interactions in a spatiotemporal manner.

**METHODS**

**Editing under Transient Expression.** 293T cells were grown in DMEM + 10% FBS + 1x P/S, 5% CO2. Plasmid transfection was done with 300 ng of the respective plasmid/well with Lipofectamine 2000 in DMEM + 1% FBS. The respective guideRNA (2.5–10 pmol/well) was reverse transfected with Lipofectamine 2000 in DMEM + 1% FBS. Cells were seeded on coverslips (DMEM + 1% FBS + HEPES). After 24 h cells were harvested for RNA sequencing or stained with BG-FITC for fluorescence microscopy as described before.

**Editing under Genomic Expression.** 293-FIp-In T-REx cells were induced in DMEM + 10% FBS + 15 μg/mL blasticidinS + 100 μg/mL hygromycinB + 10 ng/mL doxycycline, 5% CO2. The respective guideRNA (5–20 pmol/well) was reverse transfected with Lipofectamine 2000 in DMEM + 10% FBS + 10 ng/mL doxycycline. Cells were seeded on coverslips (DMEM + 10% FBS + HEPES + doxycycline). After 24 h cells were harvested for RNA sequencing or stained with BG-FITC for fluorescence microscopy.

**Light-Induced RNA Editing.** Experiments were carried out as described above with an additional irradiation step 4 h after guideRNA transfection. Cells were washed and the entire well was irradiated with 365 nm light on the microscope (Zeiss CellObserverZ1, 365 nm LED light source) for 5 s under full power at 5× magnification. Then the protocol was continued as described above.

**BG-FITC Staining.** To visualize the localization of SNAP-ADAR2, acetylated BG-FITC (final concentration 2 μM) was applied to the cells together with a blue Hoechst stain (Thermo Fisher, R37605) for 30 min. Cells were fixed with formaldehyde and permeabilized with 0.1% Triton X-100. Cover glasses were mounted using Shandon Immu-Mount (Thermo Fisher, USA).

**Immunofluorescence Microscopy.** Cells were fixed with formaldehyde and blocked with PBS + 10% FBS at 4°C overnight. Cells were stained with a primary mouse anti-HA-antibody (Sigma-Aldrich, H9658) diluted 1:1250 in PBS + 5% FBS for 1.5 h at room temperature, and a secondary antimouse antibody conjugated to AlexaFluor-594 (Thermo Fisher, A-11005) diluted 1:1500 in PBS + 10% FBS for 45 min at room temperature. Cover glasses were mounted using Dako mounting medium (Dako North America, USA). Microscopy was performed with a Zeiss CellObserverZ1 under the Zeiss CellObserverZ1 under 600x total magnification.

**ASSOCIATED CONTENT**

**Supporting Information**

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