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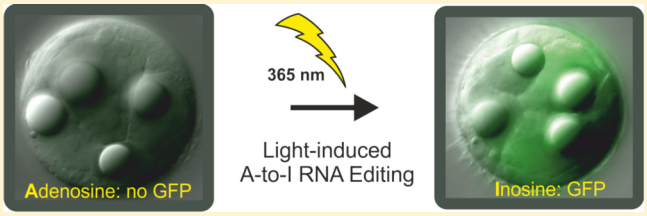
Site-Directed RNA Editing in Vivo Can Be Triggered by the Light-Driven Assembly of an Artificial Riboprotein

Alfred Hanswillemenke,[†] Tahsin Kuzdere,[†] Paul Vogel,[†] Gáspár Jékely,[‡] and Thorsten Stafforst*[,†]

[†]Interfaculty Institute of Biochemistry, University of Tübingen, Auf der Morgenstelle 15, 72076 Tübingen, Germany
[‡]Max-Planck-Institute for Developmental Biology, Spemannstraße 35, 72076 Tübingen, Germany

Supporting Information

ABSTRACT: Site-directed RNA editing allows for the manipulation of RNA and protein function by reprogramming genetic information at the RNA level. For this we assemble artificial RNA-guided editases and demonstrate their transcript repair activity in cells and in developing embryos of the annelid *Platynereis dumerilii*. A hallmark of our assembly strategy is the covalent attachment of guideRNA and editing enzyme by applying the SNAP-tag technology, a process that we demonstrate here to be readily triggered by light in vitro, in mammalian cell culture, and also in *P. dumerilii*. Lacking both sophisticated chemistry and extensive genetic engineering, this technology provides a convenient route for the light-dependent switching of protein isoforms. The presented strategy may also serve as a blue-print for the engineering of addressable machineries that apply tailored nucleic acid analogues to manipulate RNA or DNA site-specifically in living organisms.



INTRODUCTION

RNA-guided machineries provide highly selective and rationally programmable tools for the site-specific manipulation of nucleic acids. Several endogenous riboproteins are known that are steered toward their endogenous targets by nucleic acid hybridization and that are readily re-addressed toward new targets by expression or administration of artificial external guideRNAs. Those include the snoRNA-guided 2′-*O*-methylation[1] and pseudo-uridinylation[2] machineries and the microRNA-guided RNA-induced silencing complex. The harnessing of the latter machinery, better known as RNA interference,[3] has developed into a standard tool in cell biology. Besides harnessing endogenous eukaryotic machineries, the engineering of artificial riboproteins for the site-specific manipulation of nucleic acids comes more and more into focus now. Tools are highly desired that simplify genetic engineering[4] and that help to elucidate the role of point mutations and RNA modifications.[5−7] Besides their application in basic biology research, such tools have potential for translation into individualized medicine. A highly topical example is the re-engineering of the bacterial CRISPR-Cas9 system for site-selective genome editing in eukaryotic cells.[8]

Endogenous riboproteins are typically assembled by molecular recognition between specific protein and RNA structures.[9] The formation of a single covalent bond between an RNA and a protein component, however, is virtually unknown for that purpose. Nevertheless, we could recently demonstrate the assembly and functioning of highly selective adenosine (A)-to-inosine RNA editing machineries inside living cells following the latter approach.[10,11] Since inosine is biochemically read as guanosine (G), editing formally creates A-to-G point mutations at the RNA level. If RNA editing is directed to the open reading frame, 12 out of the 20 canonical amino acids can be substituted,[12] including most of the polar residues essential for enzyme catalysis, post-translational protein modification, or signaling. Furthermore, editing in the non-coding part of the RNA can interfere with translation initiation (start codon), translation stop, microRNA action, and splicing among others.[13,14] Thus, the potential of site-directed RNA editing for application in basic biology research and medicine is evident.[15−18]

We apply the SNAP-tag technology[19] to assemble the editing machinery via covalent bond formation. This technology requires the fusion of a SNAP-tag domain (an evolved *O*6-alkylguanine-DNA alkyltransferase) with the C-terminal catalytic domain of a human ADAR enzyme (adenosine deaminases acting on RNA).[10] At the RNA component, the incorporation of a small chemical moiety, *O*6-benzylguanine (BG), is necessary. The covalent bond is then formed in situ in a single-turnover enzymatic reaction between the SNAP-tag and the BG moiety with very fast kinetics ($k_{conjugation} = 2.8 \times 10^4$ M^{-1} s^{-1})[20] and high specificity (Figure 1a). Recently, we demonstrated the repair of a premature stop codon (UAG) into a tryptophan codon (UIG) in a fluorescent reporter gene in human cells (293T).[11] Notably, the repair reaction was strongly dependent on the covalent attachment of the guideRNA to the deaminase. This opens the appealing possibility of controlling the editing reaction by triggering the assembly of the covalent RNA−protein conjugate (Figure 1a). We decided to apply light as a trigger, as it allows for the very precise and fast control in time, space, and dosage.[21]

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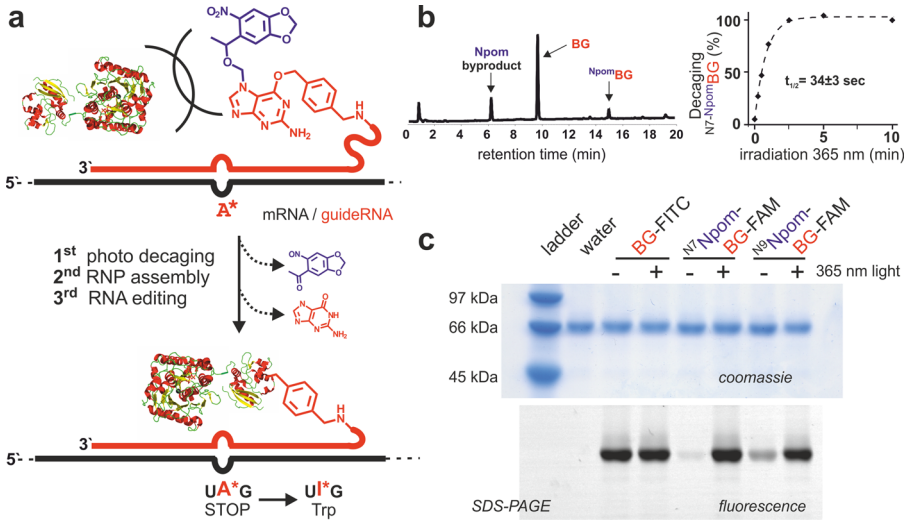

Figure 1. (a) Concept of light-triggered site-directed RNA editing. Assembly of the guideRNA–deaminase conjugate requires release of the Npom-protected benzylguanine (BG) moiety and is a prerequisite for the editing reaction. (b) First-order kinetic analysis (via HPLC) of the photodeprotection of N7-^{Npom}BG at the small-molecule level. The HPLC trace shows the product mixture after 60 s of 365 nm irradiation (75% conversion). The respective analysis for N9-^{Npom}BG can be found in Figure S13. (c) Light-triggered conjugation reaction of fluorescein-labeled Npom-BG with SNAP-ADAR1 protein (SDS-PAGE coomassie versus fluorescein stain). BG-FITC refers to the conjugate of BG with fluorescein isothiocyanate, and BG-FAM refers to the conjugate with 6-carboxyfluorescein.

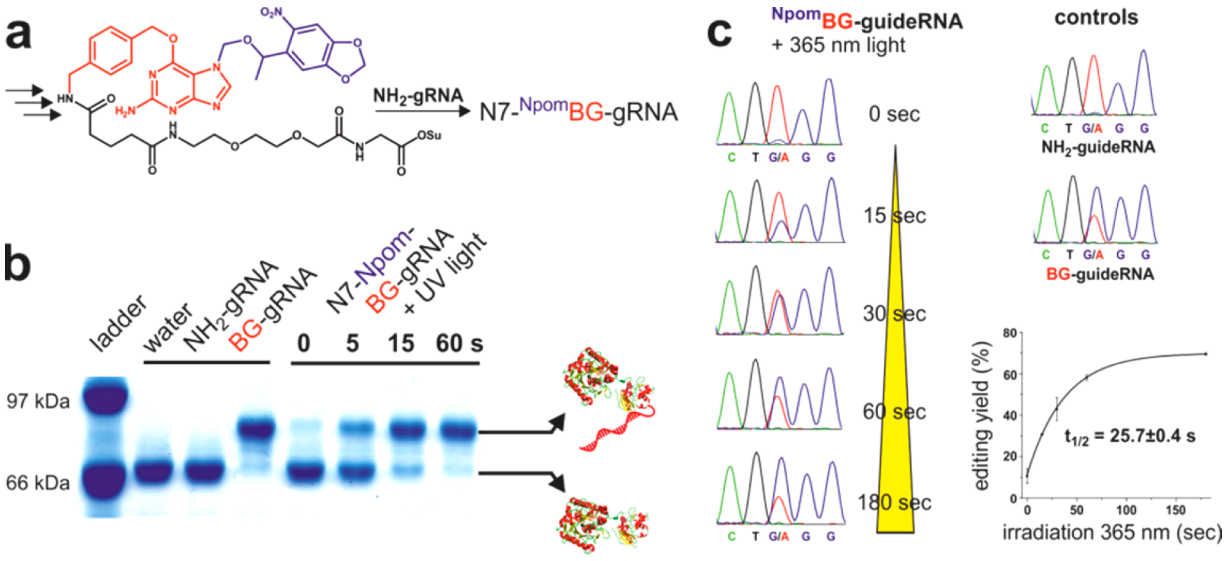

Figure 2. Light-dependent assembly of the editase and in vitro RNA editing. (a) N7-^{Npom}BG is included into an activated linker that readily reacts with the aminolinker of commercially available RNA analogues to obtain the ^{Npom}BG-guideRNAs. (b) The light-driven conjugation reaction between Npom-BG-guideRNA and SNAP-ADAR1 is easily monitored by SDS-PAGE (coomassie stain). (c) In vitro site-directed RNA editing of the amber stop codon at position 66 in the eCFP gene. The editing yield is clearly light-dose-dependent obeying first-order kinetics. Sanger sequencing of the entire ORF of the eCFP gene shows no off-target editing (Figure S15). The respective editing applying SNAP-ADAR2 instead of SNAP-ADAR1 is given in Figure S16. For further details, see the Supporting Information.

RESULTS AND DISCUSSION

Synthesis and Decaging of Npom-Protected *O*6-Benzylguanine. To achieve the light-dependent assembly of the covalent RNA–protein conjugate, we masked the BG moiety chemically by installment of a light-sensitive 6-nitropiperonyloxymethyl (Npom) protection group[22,23] which absorbs broadly in the 330–420 nm range. During synthesis we obtained a separable 1:2 mixture of regioisomers containing the Npom group either at N7 or N9 position of the guanine base. Upon irradiation with 365 nm light on a common UV-light table, both isomers, N7 and N9, decay efficiently into free BG and the respective nitroso acetophenone byproduct with similar kinetics (N7 isomer, Figure 1b, $t_{1/2}$ = 34 ± 3 s; N9 isomer, Figure S13, $t_{1/2}$ = 47 ± 4 s). The decaging efficiency $\varepsilon\phi$ was determined by comparison with a commercial standard (DMNB-cAMP) to be ~2000 and ~1500 M^{-1} cm^{-1} for the N7 and N9 isomers, respectively, giving quantum yields $\phi \approx$ 0.5 and 0.36 (for details, see the Supporting Information).

To determine the reactivity of the Npom-protected BG with SNAP-deaminases, we modified the aminomethyl linker of the BG moiety with fluorescein. Such probes were incubated with sub-stoichiometric amounts of SNAP-ADAR1 either in the dark or in the presence of 365 nm light. The conjugate formation was then determined by SDS-PAGE and fluorescence analysis

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(Figure 1c). It was clearly shown that full fluorescence labeling of the SNAP-deaminase was readily accessible upon irradiation. However, some background reactivity of the protected BG in particular of the N9 isomer with SNAP-ADAR1 was visible. The latter is coherent with the requirement of the natural ancestor of the SNAP-tag to accept the desoxyribose at the N9 position of the nucleobase.[24] We did not expect the low-level residual activity to play a role under dilute conditions inside the living cell; nevertheless, we continued all further work with the pure N7 isomer of Npom-BG.

Light-Triggered Assembly of Protein−RNA Conjugates Controls RNA Editing in Vitro. To study the assembly of the guideRNA−deaminase conjugate and its effect on in vitro RNA editing, we attached the N7-^{Npom}BG via a short linker[10] to the 5′-terminal aminolinker of a 17 nt guideRNA that directs the conjugate to codon 66 of the eCFP transcript in order to stimulate the repair of a premature amber stop codon (UAG) back to tryptophan (Figure 2a). Via SDS-PAGE we first characterized the light-dependent assembly of the riboprotein (Figure 2b). Conjugation results in a readily detectable shift of the SNAP-deaminase toward higher molecular weight. Indeed, excellent control of the conjugate assembly was achieved in a clearly light-dose-dependent manner, shifting the SNAP-deaminase from non-conjugated to nearly complete conversion following kinetics agreeing with the decaging kinetics of the ^{Npom}BG precursor described above. To study the light-dependent in vitro RNA editing reaction, a master mix containing all components was aliquoted in the dark into PCR tubes and aliquots were irradiated individually with 365 nm light for 0, 15, 30, 60, or 180 s, respectively, prior to starting the editing reaction by incubation at 37 °C. A guideRNA lacking the BG moiety served as a negative, and a guideRNA modified with authentic BG served as a positive control. No editing was observed in the negative control. Similarly, only very minor editing above background was detectable in the non-irradiated sample with the Npom-caged BG-guideRNA. However, upon irradiation editing was restored in a light-dose-dependent manner regaining an editing level comparable to that of the positive control (Figure 2c, 75% with SNAP-ADAR1; Figure S16, 60% with SNAP-ADAR2). Plotting the intermediate editing levels against the irradiation time resulted in first-order kinetics (Figure 2c, $t_{1/2}$ = 26 ± 0.5 s) very similar to those obtained with the small-molecule precursor (Figure 1b).

RNA Editing Is Controllable by Light in Living Cells. For the study of intracellular light-activated RNA editing, we incorporated the N7-^{Npom}BG into a 19 nt Antagomir-like[25] chemically stabilized nucleic acid analogue[11] that contained a gap of three natural ribonucleotides around the editing site. We applied Antagomir-like chemistry to improve the stability of the guideRNA and the selectivity of the editing reaction;[11] this has been shown for RNA interference before.[26] The guideRNA targets a premature amber stop codon (UAG) at an eGFP reporter (W58amber), and successful editing is indicated by turn-on of eGFP fluorescence. A guideRNA with authentic BG served as a positive and the same guideRNA lacking the BG moiety as a negative control. Further controls were done to test the necessity of all components of the machinery. SNAP-ADAR1 and the reporter gene were transiently overexpressed from plasmids in HEK293T cells. One day after transfection of the plasmids, the respective guideRNA was lipofected into the cells. Four hours after lipofection, the medium was changed and cells were irradiated with 365 nm light under high control of dosage and wavelength by using a fluorescence microscope equipped with a LED light source. One day later, the eGFP fluorescence was analyzed by microscopy before the RNA was isolated, and the editing yield was determined by Sanger sequencing. Compared to our previous protocol, we had to optimize the amounts and stoichiometry of SNAP-ADAR1 and guideRNA in order to suppress some low-level (∼10%) editing caused by the Npom-protected guideRNA in the dark (for details, see Figures S19 and S20). The optimal amount of guideRNA used was 10 pmol/150 μL and is in a range typical for siRNA duplexes. Applying the NH_2-guideRNA (negative editing control), only a very few cells developed a low-level GFP fluorescence and no editing was detectable in the sequencing trace (<5%). However, transfecting BG-guideRNA (positive editing control) gave brightly fluorescent cells, similar to the transfection of functional wt eGFP, and an editing yield of typically 45% was determined (Figures 3 and S17). Notably,

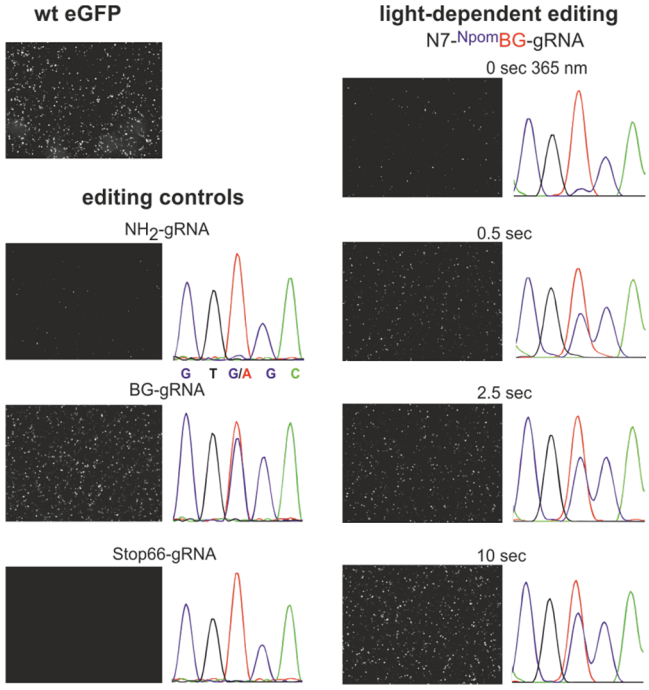

Figure 3. Light-controlled RNA editing in living 293T cells. SNAP-ADAR1 and the reporter gene (W58X eGFP, or wt eGFP) are provided on plasmids, the guideRNAs are reverse-transfected, all as described in the Supporting Information. Shown is the fluorescence microscopy analysis together with the respective Sanger sequencing traces 24 h post-transfection of the respective guideRNA. 5′-Terminal, the guideRNAs are either carrying an aminolinker (NH_2), the BG moiety (BG), or the Npom-protected BG moiety. The Stop66-guideRNA is a negative control BG-guideRNA targeting the GFP gene around codon 66 instead of codon 58.

no other edited site was observed in the reporter transcript. Furthermore, absolutely no editing was obtained at codon 58 by a chemically stabilized, negative control guideRNA that directs repair to codon 66. Thus, the formation of the RNA secondary structure directly at the targeted codon is strictly required for site-directed RNA editing and is the major determinant of specificity. The Npom-protected guideRNA gave only very low editing yield over background (<5%) and only a small number of low-intensity fluorescent cells. However, following illumination, editing was switched on to a level similar to that of the positive editing control, as indicated

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by fluorescence microscopy but also by RNA sequencing (45% editing yield). Intermediate editing levels have been accessible by varying the light dose (Figure 3; more details can be found in Figure S17). The light dose applied to photoactivate editing was well tolerated by the cells. In comparison to the Npom-guideRNA, the editing yield of neither the positive nor the negative editing controls was dependent on light (Figure S17). No unspecific off-target editing was observed in the reporter gene, as indicated by Sanger sequencing (Figure S18).

Site-Directed RNA Editing in *Platynereis dumerilii*. As it requires massive genetic manipulation to switch the expression of one isoform to another that differs only in a single point mutation, site-directed RNA editing might offer a practical alternative.[15] A light-triggered variant would be particularly attractive for developmental biology, as early stages are often transparent,[21] and the spatiotemporal control of gene expression is of particular interest.[27,28] An emerging model system for developmental and neurobiology is the marine annelid *Platynereis dumerilii*[29] that is readily cultivated[30] and easily manipulated at the one-cell zygote by microinjection.[27] To test site-directed RNA editing inside the worm, we injected two mRNA transcripts encoding SNAP-ADAR1 and eGFP together with chemically stabilized 21 nt guideRNAs. One day after microinjection, when the zygotes were developing into trochophore larvae, the fluorescence phenotype was analyzed by microscopy (Figures 4 and S22). A GFP-positive phenotype was only detectable in the positive control (wt GFP) and in the editing sample (Figure 4a,f). All negative controls lacking parts of the machinery, such as the guideRNA, SNAP-ADAR1, or both, showed no green fluorescence (Figure 4b–d). In the editing sample as well as in the positive control, there was some heterogeneity of fluorescence intensity that may result from the difficulty of precisely controlling the injection volume. To determine the editing yield, a cohort of trochophores (each 80–100) were lysed, and RNA was extracted and analyzed by Sanger sequencing (Figure 4; for detailed analysis of all larvae, see Table S23). Editing was observed only when all components were included and achieved 60–70% over the entire population. No off-target editing was observed in the targeted transcript (Figure S24). The worms seem to develop and behave normally over the first days and stages of development.

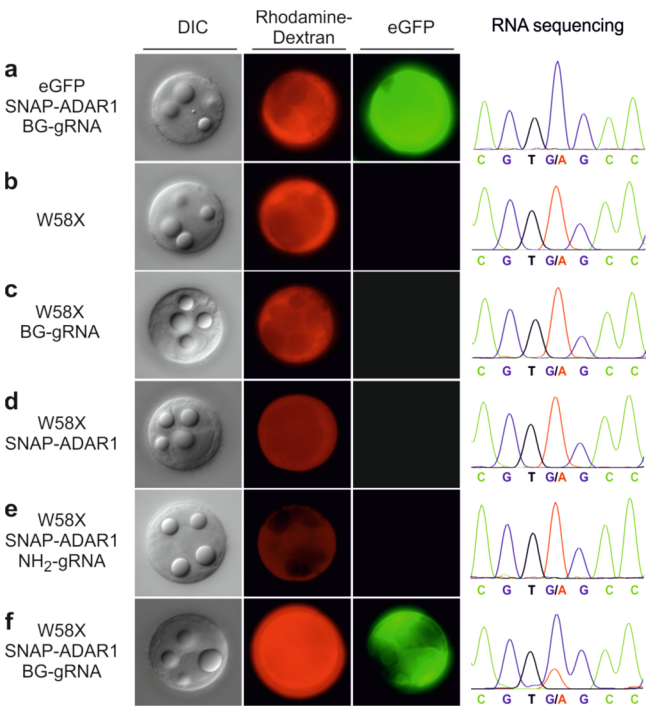

Figure 4. Site-directed RNA editing in *P. dumerilii*. Reporter mRNA (eGFP) and SNAP-ADAR1 mRNA were microinjected into one-cell zygotes, together with the respective BG/NH_2-guideRNA and rhodamine–dextran as an injection control. Shown are the fluorescence images of one representative embryo 24 hours post fertilization (hpf) for each experiment and the sequencing trace obtained from the RNA of 80–100 animals per experiment: (a) positive control, (b–e) negative controls lacking single components of the editing machinery, and (f) editing experiment. For details, see the Supporting Information. DIC = differential interference contrast.

Controlling Site-Directed RNA Editing in Living *P. dumerilii* by Light. Also in *Platynereis*, efficient editing requires assembly of the covalent guideRNA–deaminase conjugate and fails when using the NH_2-guideRNA lacking the BG moiety (Figure 4e). This encouraged us to test light-activated RNA editing inside the worm. For this, a guideRNA containing the Npom-protected BG was microinjected. In contrast to using the NH_2-guideRNA (Figure 4e) lacking the BG moiety, microinjection of the Npom-protected guideRNA resulted in a small but significant number of faintly fluorescing trochophores (18%) besides a large number of dark ones (>80%, Figure 5a, Table S23). In accordance with this, RNA sequencing of a cohort of 80–100 animals revealed a low but significant residual editing at the targeted stop codon (~10%). In faintly fluorescent trochophore larvae, the rhodamine signal was typically stronger (Figure 5a), indicating that the low-level editing might be due to an undesirably high injection volume of the editing components. This low-level residual editing activity is reminiscent of the situation described above for the light-dependent editing in cell culture. However, when the microinjected trochophores were treated with 365 nm light on a UV trans-illuminator (5 min), half of the trochophores developed a bright eGFP signal (Figure 5b, Table S23). The fluorescence imaging was in agreement with an editing yield of ~60%, as determined by RNA sequencing of 80–100 animals (Figure 5b). Thus, irradiation allows for activating RNA editing to a yield nearly identical to that of the positive editing control with an unprotected BG moiety (Figure 4f).

CONCLUSION

RNA-guided enzymes represent rationally programmable tools that allow for the efficient and precise manipulation of nucleic acids at specific sites in living organisms. Here, we further elaborate a novel strategy for site-directed adenosine-to-inosine RNA editing (a) by introducing photocontrol and (b) by applying the tool in developing *Platynereis dumerilii*.

The presented approach is unique in that the artificial RNA-guided editing enzyme is assembled via the formation of a single covalent bond.[10,15] As covalent bond formation is essential for the functioning, photocontrol is feasible by blocking the SNAP-tag-mediated bond formation via installment of a single photoprotection group at the *O*6-benzylguanine moiety. Specifically, we demonstrate the ready synthesis of Npom-protected BG and its convenient introduction into diversely chemically modified antisense oligomers after their solid-phase synthesis. This is in contrast to other strategies that require the site-specific incorporation of (often several) photoprotected nucleosides during solid-phase

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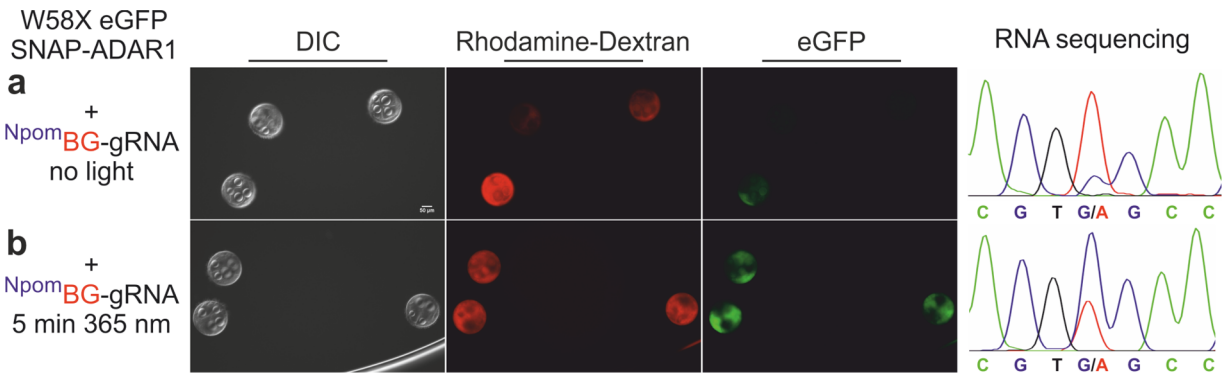

Figure 5. Light-dependent editing in *P. dumerilii*. Reporter gene and SNAP-ADAR1 have been microinjected into one-cell *Platynereis* zygotes as described in Figure 4, but now with a photoprotected chemically stabilized ^{Npom}BG-guideRNA. Within 1 h after microinjection, zygotes have been (a) kept in the dark or (b) treated with 365 nm light (5 min). Fluorescence images and RNA sequences (80−100 animals/experiment) are taken 24 hpf. The sequence of the guideRNA is the same as in Figure 4. For further details, see the Supporting Information.

oligonucleotide synthesis to achieve photocontrol of biochemical processes.[21,23,31] Furthermore, our strategy needs less genetic engineering compared to the introduction of photoresponsive groups into enzymes by means of amber suppression or related strategies.[32,33]

In vitro we could show that the attachment of the Npom group at N7, but not N9, of the BG moiety blocks the conjugation reaction with the SNAP-tag. However, reactive benzyl guanine is readily released upon 365 nm irradiation with high efficiency ($\varepsilon\varphi \approx 2000\ M^{-1}\ cm^{-1}$) and allows for the light-dose-dependent assembly of guideRNA−deaminase conjugates. Besides editing, the Npom-protected BG will be applicable in other approaches that rely on the SNAP-tag, like chemical inducers of dimerization.[34] By controlling the assembly of the editase, we could trigger the in vitro editing of a purified mRNA in a light-dose-dependent manner covering the whole dynamic range from absence of editing in the absence of light until full editing in the presence of light. The desired action of our tool could be directly translated into mammalian cell culture; however, optimization was required to control low-level residual editing by the photoprotected guideRNA. Again, a similarly high dynamic range was achieved. Furthermore, we established site-directed RNA editing for the first time in a living organism. Specifically, we achieved the efficient and highly selective switch of a premature stop into a tryptophan codon in developing *Platynereis dumerilii* zygotes. Notably, no genetic engineering and livestock breeding is required, thus circumventing time-consuming and cost-intensive laboratory work. As our editing tool is independent of any host-specific factors, the technology should be transferable to any other organism. In *Platynereis*, the covalent assembly of the guideRNA−deaminase conjugate was again essential, and our simple photocontrol strategy for site-directed RNA editing was directly transferable. The tool could now be further elaborated to achieve precise spatiotemporal control of protein isoforms in cellular networks or in developing *Platynereis*.

MATERIALS AND METHODS

Synthesis of Npom-Caged *O6*-Benzylguanine. Trifluoroacetamide protected *O6*-benzylguanine (BG, 120 mg, 0.33 mmol)[19] was solved in dry DMF (1.2 mL) under argon. Diazabicycloundecene (150 μL, 153 mg) was added at room temperature, and the solution was stirred for 30 min. Npom chloride (0.5 mmol, ∼1.5 equiv, dissolved in 1.6 mL of DMF) prepared *in situ* as described[22] was added dropwise. After 2.5 h, the reaction mixture was diluted with EtOAc, washed with 1% citric acid (3×) and brine (1×), and dried over Na_2SO_4. The evaporated crude product was cleaned via silica chromatography (2−4% MeOH in DCM) and yielded 24 mg (21%) of ^{N7}Npom-BG-TFA and 50 mg (42%) of ^{N9}Npom-BG-TFA. For full characterization and assignment of the isomers and downstream synthesis, see the Supporting Information.

N7 Isomer. ^{1}H NMR (600 MHz, DMSO-d_6): δ = 9.99 (t, J = 5.9 Hz, 1H), 8.07 (s, 1H), 7.48 (d, J = 8.1 Hz, 2H), 7.35 (s, 1H), 7.28 (d, J = 8.1 Hz, 2H), 6.84 (s, 1H), 6.22 (s, 2H), 6.16 (s, 1H), 6.04 (s, 1H), 5.54 (m, 2H), 5.46 (m, 2H), 5.13 (q, J = 6.2 Hz, 1H), 4.39 (d, J = 5.9 Hz, 2H), 1.33 (d, J = 6.2 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6): δ = 164.1, 159.8, 156.4 (q, ^2J(C,F) = 36 Hz), 156.3, 151.8, 146.5, 145.9, 140.5, 137.2, 136.2, 135.5, 127.9, 127.5, 116.0 (q, ^1J(C,F) = 288 Hz), 105.6, 105.2, 104.3, 103.2, 74.9, 72.0, 66.6, 42.4, 23.3. HR-ESI-MS: $[M + H]^+_{(theoretical)}$ = 590.16056 for $C_{25}H_{23}F_3N_7O_7$; found 590.16118. R_f(DCM/MeOH, 98:2) = 0.08. R_f(DCM/MeOH, 95:5) = 0.50.

N9 Isomer. ^{1}H NMR (600 MHz, DMSO-d_6): δ = 10.00 (t, J = 6.0 Hz, 1H), 7.80 (s, 1H), 7.49 (d, J = 8.1 Hz, 2H), 7.46 (s, 1H), 7.31 (d, J = 8.1 Hz, 2H), 6.97 (s, 1H), 6.34 (s, 2H), 6.15 (s, 1H), 6.03 (s, 1H), 5.43−5.49 (m, 2H), 5.40 (d, ^2J = 11.4 Hz), 5.32 (d, ^2J = 11.4 Hz), 5.21 (q, J = 6.3 Hz, 1H), 4.41 (d, J = 6.0 Hz, 2H), 1.38 (d, J = 6.3 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6): δ = 160.8, 157.2 (q, ^2J(C,F) = 36 Hz), 155.3, 152.7, 147.5, 142.1, 140.5, 138.2, 137.0, 136.6, 129.6, 128.3, 116.9 (q, ^1J(C,F) = 288 Hz), 114.3, 106.7, 105.2, 104.1, 73.1, 71.7, 67.5, 43.3, 24.1. HR-ESI-MS: $[M + Na]^+_{(theoretical)}$ = 612.14250 for $C_{25}H_{22}F_3N_7O_7Na$; found 612.14262. R_f(DCM/MeOH, 98:2) = 0.32. R_f(DCM/MeOH, 95:5) = 0.55.

Light-Triggered in Vitro RNA Editing. Purified SNAP-ADAR1 (170 nM), purified eCFP mRNA (10 nM), and one of the respective guideRNAs (50 nM) were prepared in buffer (25 mM Tris·HCl, 0.75 mM $MgCl_2$, 75 mM KCl, 2 μM heparin, and 640 u/mL murine RNase inhibitor, 10 mM DTT, pH 8.3) in PCR tubes. Irradiation with 365 nm light was performed on a UV trans-illuminator (UVP TFL-40V, 25 W, intensity high) for the indicated amount of time at room temperature. Subsequent editing was performed by incubation for 120 min while cycling between 30 and 37 °C. Reactions were stopped by heating to 70 °C for 3 min and subsequent reverse transcription. After PCR amplification of the cDNA, editing yields were estimated by the relative height of the guanosine versus adenosine traces by Sanger sequencing. All experiments were done in at least two replicates. Sequence of the guideRNAs: (Npom)BG/NH_2-UCG-GAACACCCC-AGCACAGA-3′ (natural ribonucleotides; 5′-terminal modifications were introduced via amino-linker, the 5′-terminal three nucleotides serve as linker and do not base-pair with the target).

Light-Triggered Cellular RNA Editing. Cells (293T: DSMZ code ACC-635; 200 000 cells/well) were seeded on 24-well plates in full media (DMEM, 10% FBS, 1% penicillin/streptomycin, grown in 5% CO_2, 37 °C). At 60−80% confluency, plasmid pcDNA3.1 vector (Life Technologies) carrying SNAP-ADAR1 (100 ng/well) and pcDNA3.1 vector carrying the respective eGFP variant (500 ng/well)[11] were co-transfected with Lipofectamine 2000 (4 μL/μg).[11]

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After 24 h, the cells were reverse transfected into 96-well plates (60 000 cells/well) containing the respective guideRNAs (10 pmol/well) pretreated with Lipofectamine 2000 (0.5 μL/well). Four hours after reverse transfection, media was replaced with DMEM without FBS and phenol red, containing HEPES (25 mM). Irradiation (365 nm) was performed in a fluorescence microscope (Zeiss CellObserverZ.1, equipped with a 365 nm Colibri.2 LED) at 100% LED power for the indicated amount of time. Twenty-four hours later, the fluorescence phenotype was analyzed by fluorescence microscopy (Zeiss CellObserverZ.1), and RNA was extracted using the RNeasy MinElute Cleanup Kit (Qiagen). After reverse transcription and PCR amplification, the editing yield was estimated by Sanger sequencing. All experiments were done in at least two replicates. The sequence of the W58X guideRNAs was (Npom)BG/NH_2-UsAsU-GUGUCGG-CCA-CGGAAsCsAsGsG-3′; the sequence of the Stop66-guideRNA was BG-UsCsG-GAACACC-CCA-GCAsCsAsGsA-3′ (s = phosphothioate linkage; plain font indicates 2′-methoxyribonucleotides, and italic underlined indicates unmodified ribonucleotides; the three 5′-terminal nucleotides serve as a linker and do not base-pair with the target).

Editing in *Platynereis dumerilii*. For the in vitro transcription of stabilized mRNAs of SNAP-ADAR1 and eGFP variants with the mMESSAGE mMACHINE T7 Ultra Kit (Life Technologies), the respective genes were subcloned into the pUC57-T7-RPP2 vector, resulting in 5′-capped and 3′-polyadenylated transcripts additionally stabilized by a *Platynereis*-specific RPP2 5′-UTR, as described before.[35] mRNA transcripts were cleaned by the RNeasy MinElute Cleanup Kit (Qiagen). GuideRNAs were precipitated with potassium acetate prior to use. Fertilized zygotes were obtained from an in-house breeding culture and were incubated at 14.8 °C for 55 min. Prior to microinjection, the egg jelly was removed by rinsing the zygotes with natural seawater (NSW) in a 100 μm sieve. To soften the vitellin envelope, a 1-min-long proteinase K treatment (70 μg/mL) was performed as described before.[27] Around 100 zygotes were embedded in the injection stage (2% agarose in NSW). Samples were injected using Femtotipps II microcapillaries with a Femtojet express microinjector (700 hPA injection pressure, 0.1 s injection time, 35 hPa compensation pressure) in a cooled (14.8 °C) Zeiss Axiovert 40 CL microscope equipped with a Luigs and Neumann micromanipulator as described before.[27] Injection started 1 hours post fertilization (hpf) and was stopped when the first cleavage was detected (ca. 2 hpf). Irradiation at 365 nm was performed immediately after microinjection for the indicated amount of time on a UV transilluminator (UVP TFL-40V, 25 W, intensity high). Microinjected zygotes were bred at 19 °C in Nunclon six-well plates containing 6 mL NSW. Twenty-four hpf, healthy larvae (early trochophore) were separated from unhealthy ones. The fluorescence phenotype was analyzed by microscopy (Axio Imager Z1). RNA from 80−100 healthy larvae (two injection sessions) was isolated 25 hpf by shock freezing (liquid nitrogen), shear forces (passing through 0.6 mm needle), vortexing (10 s), and subsequent use of the RNeasy MinElute Cleanup Kit (Qiagen). After reverse transcription and PCR amplification, the editing yield was determined by Sanger sequencing. Injection samples contained 1.5 μg/μL rhodamine-dextran (10 kDa MW, Sigma) for injection control, 250 ng/μL of the respective reporter mRNA, 450 ng/μL SNAP-ADAR1 mRNA, and 25 μM of the respective guideRNA. Sequence of the guideRNA: BG/NH_2-UsAsU-GUGUCGG-CCA-CGGAACAsGsGsCsA-3′ (s = phosphothioate linkage; plain font indicates 2′-methoxy ribonucleotides, and italic underlined indicates unmodified ribonucleotides; the 5′-terminal three nucleotides serve as linker and do not base-pair with the target).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b10216.

Chemical synthesis, compound characterization, molecular biology, and editing experiments, including Figures S1−S26 and Tables S1−S23 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*thorsten.stafforst@uni-tuebingen.de

Notes

The authors declare no competing financial interest.

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