

Small Molecule-Inducible RNA-Targeting Systems for Temporal Control of RNA Regulation

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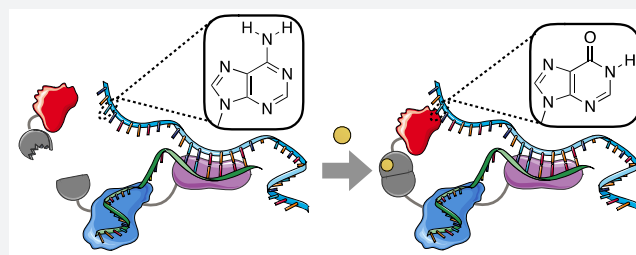
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ABSTRACT: All aspects of mRNA lifetime and function, including its stability, translation into protein, and trafficking through the cell, are tightly regulated through coordinated post-transcriptional modifications and interactions with a multitude of RNA effector proteins. Despite the increasing recognition of RNA regulation as a critical layer of mammalian gene expression control and its increasing excitement as a therapeutic target, tools to study and control RNA regulatory mechanisms with temporal precision in their endogenous environment are lacking. Here, we present small molecule-inducible RNA-targeting effectors based on our previously developed CRISPR/Cas-inspired RNA targeting system (CIRTS). The CIRTS biosensor platform is based on guide RNA (gRNA)-dependent RNA binding domains that interact with a target transcript using Watson–Crick–Franklin base pair interactions. Addition of a small molecule recruits an RNA effector to the target transcript, thereby eliciting a local effect on the transcript. In this work, we showcase that these CIRTS biosensors can trigger inducible RNA editing, degradation, or translation on target transcripts in a small molecule-dependent manner. We further go on to show that the CIRTS RNA base editor biosensor can induce RNA base editing in a small molecule-controllable manner *in vivo*. Collectively this work provides a new set of tools to probe the dynamics of RNA regulatory systems and control gene expression at the RNA level.



INTRODUCTION

Gene expression regulation at the RNA level comprises a complex set of mechanisms that control the type, amount, and location of protein production within a cell. RNA turnover, for example, varies greatly between different transcripts, which in turn regulates the resulting gene expression levels.^{1,2} Approaches to alter the RNA abundance and lifetime, such as RNAi, are not only powerful research tools but are also finding significant therapeutic value.^{3,4} In addition, translation of individual RNAs into protein is tightly regulated from the initiation to the termination steps.⁵ Aside from the regulation of lifetime and translation, the localization of an RNA is also a critical aspect of gene expression control at the RNA level.⁶ For example, in neurons, RNAs transcribed in the nucleus need to first be properly trafficked to a synapse where they are locally translated into synaptic proteins.^{7,8} Each of these regulatory layers are coordinated by a suite of protein–RNA interactions that collectively tune gene expression.

Apart from canonical RNA processing mechanisms, such as capping, splicing, polyadenylation, and trafficking, RNA sequences can be edited and chemically modified.^{9–12} For example, adenosine-to-inosine (A-to-I) editing is a common post-transcriptional chemical modification, modulated by the hADAR family of proteins, which deaminate A in double-stranded RNA (dsRNA) at the site of a C–A mismatch.^{13,14} The deamination product, inosine, mimics guanosine (G) in cells by base-pairing with cytosine (C), allowing for both

coding amino acid and splicing changes.^{15–17} In addition to RNA editing, RNA bases can be chemically modified. N⁶-Methyladenosine (m⁶A) is the most prevalent internal RNA modification in mammalian systems.¹⁸ m⁶A is dynamically installed and removed by writer and eraser enzymes, respectively, and is linked to cellular functions by recognition and binding of m⁶A reader proteins.^{19,20} RNA chemical modifications influence RNA stability, splicing, export, and translation efficiency^{21–25} and have been linked to various cancers and viral infections.^{26–28} With increasing numbers of regulatory pathways influencing RNA lifetime being uncovered, precisely controllable technologies are needed to study the temporal dynamics of these processes in their endogenous environment.

Programmable RNA-targeting tools that allow for site-specific RNA targeting hold great promise for studying biological processes in an endogenous context as well as for therapeutic applications. For example, Cas13 proteins of the CRISPR–Cas system have been developed to deliver a variety

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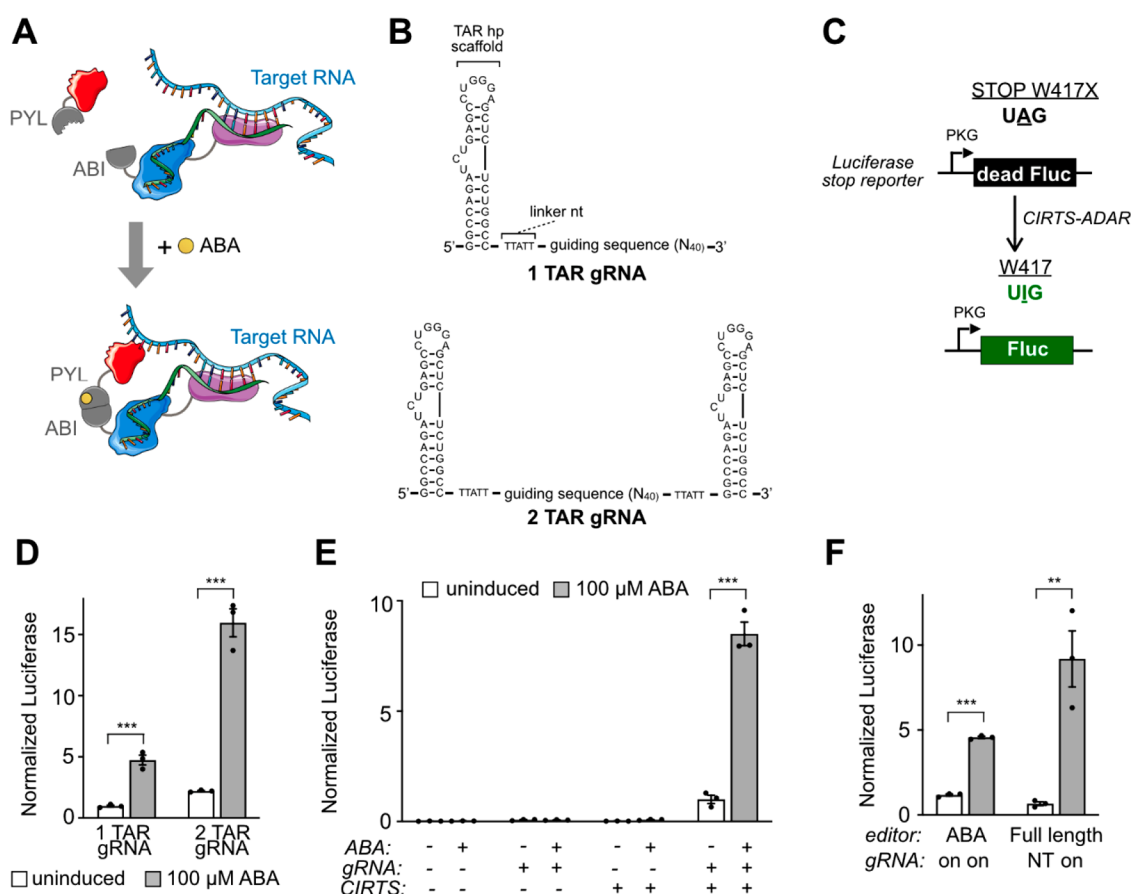


Figure 1. Development of a CIRTs biosensor. (A) Schematic of the small molecule-inducible CIRTs biosensor. (B) Design of the guiding RNA (gRNA) containing one or two hairpin sequences. (C) Schematic of stop codon reversion luciferase assay. Stop codon reversion should only be observed when ABA is added to assemble a fully functional base editor at the target site. (D) gRNA comparison of the original 1 TAR gRNA with a 2 TAR gRNA. HEK293T cells transfected with a vector expressing each half of the CIRTs base editor and a vector expressing a gRNA targeting the luciferase reporter using one of the two structures shown in B. Twenty-four h after induction with ABA, luciferase in uninduced and induced samples was measured. (E) Cells treated as in D, with individual components of the biosensor to verify that the observed editing is a result of the targeted, induced CIRTs biosensor. Robust editing was only observed when all CIRTs components were present and ABA was added. (F) Comparison of ABA-inducible CIRTs-hADAR2(E488Q) to full length CIRTs-hADAR2(E488Q) in cells as treated in D. All values are mean $\pm$ SEM with $n = 3$ biological replicates. Student t test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of effector proteins, including RNA editors,^{29,30} splicing modulators,³¹ and translational activators to RNA transcripts and sites of interest.³² We recently developed CIRTs as a smaller, human-derived delivery moiety for RNA effectors.³³ CIRTs relies on guide RNA (gRNA) complementarity to bind a target of interest and deliver a tethered cargo protein. While both Cas13 and CIRTs-based tools can deliver RNA regulatory proteins to a transcript and site of interest, they offer no temporal control to study effector dynamics. Indeed, to address this need, conditional control technologies using masked nucleic acids that can be chemically- or light-activated have been developed for Cas9 and Cas13.^{34–36} For ribonucleoprotein-based DNA targeting, inducible technologies, primarily based on the Cas9 protein family, have been developed and provide valuable tools to probe genetic regulation.^{37,38} However, comparable systems for transcriptomic regulation are just starting to be developed³⁹ and are largely still lacking. Herein, we describe the development of a small molecule-inducible RNA-targeting system based on CIRTs that allows for specific temporal control of RNA regulation. We first show that the CIRTs biosensor is a versatile small molecule-inducible platform by demonstrating inducible RNA editing, degradation, and translation activation

in cell culture models. We then go on to demonstrate that the CIRTs biosensor can be used *in vivo* to induce RNA effectors in a small molecule-dependent manner. Taken together, we demonstrate the development and first applications of a versatile, small molecule-controllable RNA effector system that allows for site- and transcript-specific studies of RNA regulation dynamics in an endogenous context.

RESULTS

Development of a Small Molecule-Inducible CIRTs Biosensor. To engineer a small molecule-inducible RNA-targeting system, we coupled CIRTs with the heterodimerization domains of the abscisic acid (ABA) system that relies on rapid binding of heterodimerization domains (ABI and PYL) in the presence of abscisic acid.⁴⁰ We chose the ABA system because it has been successfully applied in Cas9-guided DNA targeting systems for inducible transcription activation of genes of interest,³⁸ and because ABA (unlike the more commonly used rapamycin dimerization systems) has limited endogenous targets in mammalian systems.⁴¹ We envisioned coupling the targeting component of CIRTs (a single-stranded RNA binding protein and hairpin binding protein) to ABI (CIRTs-ABI) and fusing the RNA effector domain to PYL

(Figure 1A). The targeting CITS component binds to a gRNA and engages the target RNA by base pair complementarity. The effector domain can then be recruited by addition of the small molecule ABA. Once added, ABA binds to PYL and triggers a conformational change enabling PYL-ABI complex formation, effectively recruiting the effector domain to the targeting moiety of CITS to elicit the desired function on the targeted RNA transcript.

To validate the ABA biosensor, we designed a luciferase reporter assay in which a single G-to-A mutation encodes a premature stop codon in luciferase mRNA that can be reverted by targeted A-to-I editing with an on-target gRNA (Figure 1B,C). We then designed a small molecule-inducible CITS editor by constructing a combined CITS-ABI and PYL-hADAR2(E488Q) vector in order to minimize the amount of transfected plasmid DNA needed and therefore simplify applications of our system. We tiled gRNAs along the reporter transcript to identify the ideal mismatch position for editing within the gRNA-target RNA duplex (Figure S1A,B) and determined a mismatch 15nt into the guiding sequence yielded the best editing efficiency. As expected, the CITS base editor was able to induce the defined edit on the reporter transcript in a small molecule-dependent manner (Figure 1D, Figure S1C). However, because the efficiency of the system was relatively low, we next sought to improve the CITS design by testing different concentrations of each biosensor component and making alterations to the gRNA structure.

To investigate the optimal expression of targeting CITS-ABI to PYL-hADAR2(E488Q), we cotransfected cells at various ratios of both biosensor components. We did, however, not observe significant differences in editing suggesting the relative expression of the targeting and effector pieces do not affect editing efficiency (Figure S1D). We therefore returned to our combined one vector biosensor plasmid mentioned above. In previous studies, it has been reported that editing systems are more efficient when the gRNA contained two hairpin sequences.^{42,43} We therefore tested whether the addition of a second TAR hairpin to the gRNA design (Figure 1B, bottom) would improve the performance of our biosensor. Indeed, we observed a significant increase in editing efficiency using the “2 TAR” hairpin design (Figure 1D), leading us to adopt this improved gRNA design for all subsequent studies. To control for gRNA-dependent editing due to endogenous ADAR, we performed control experiments, delivering each individual component of our system in isolation. We observed some low levels of background editing when only the on-target gRNA was delivered and when we deliver the on-target gRNA and both overexpressed components of the editing system in the absence of ABA (Figure 1E and Figure S1E). We anticipated this finding as the gRNA will create the needed double-stranded region for efficient hADAR editing, and we simultaneously overexpressed the editing enzyme. However, we only observed robust, significant editing when we transfected cells with the ABA-CITS biosensor and gRNA and induced the system with ABA (Figure 1E). We then compared the biosensor CITS editor to the full-length CITS editor and found that conversion of CITS into a biosensor only results in a 2-fold loss of efficacy (Figure 1F).

Time-Dependent RNA Editing Using CITS Base Editors. After validating our inducible CITS biosensor, we assayed the temporal dynamics of the inducible editing response. We conducted an ABA time course experiment and measured the resulting RNA editing levels for 3 days.

Editing efficiency was monitored by both a cell-based luciferase stop codon reversion assay (Figure 2A) and by quantifying

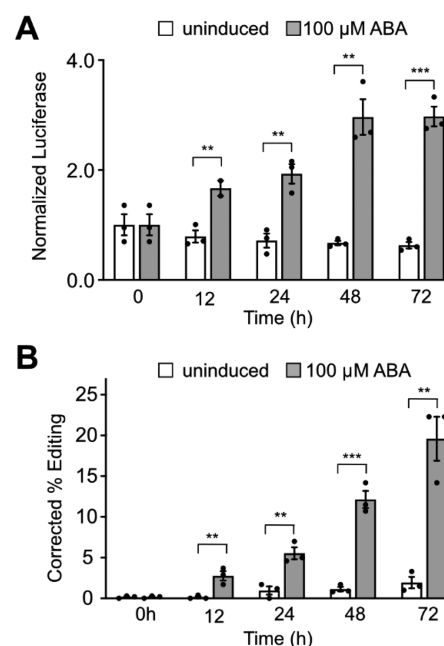


Figure 2. Temporal dynamics of the inducible editing response. (A) Time course with CITS editor for 3 days after ABA addition as quantified by stop codon reversion luciferase assay. HEK293T were transfected with a vector containing the CITS biosensor and a gRNA expression vector. Twenty-four h after transfection, the CITS biosensor was induced by addition of ABA, and editing levels were monitored for 72 h. (B) After conducting the same experiment as detailed in A, RNA editing percentage was directly quantified by RT-PCR-Sanger sequencing. All values are mean $\pm$ SEM with $n = 3$ biological replicates. Student t test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RNA edits directly using reverse transcription (RT)-polymerase chain reaction (PCR) followed by Sanger sequencing (Figure 2B) and a correction for fluorophore and PCR biases with a standard curve (Figure S2A,B). Both G content at the mutation site and luciferase readout increase steadily over the course of 72 h of ABA-induced editing via our CITS biosensor (Figure 2). We verified the Sanger sequencing quantification method by comparing the results obtained by quantifying samples by Sanger and high-throughput sequencing and found that the measured editing levels were comparable (Figure S2C). Lastly, we set out to assess the dynamics of biosensor resetting after removal of ABA. We treated cells with ABA for 24 h to induce editing before removing the small molecule and monitored the remaining luciferase signal over 3 days using the luciferase reporter assay. Luciferase activity remained unchanged for 24 h before completely returning to baseline activity over 48 h (Figure S2D). We attribute the lag in luciferase signal decrease to the half-life of the luciferase protein within cells and anticipate more rapid decay dynamics when monitoring RNA levels directly. Nevertheless, we showcased the reversible nature of our CITS editing biosensor as a tool to study RNA effector protein dynamics in their endogenous environment.

Inducible Editing of Endogenous and Disease-Relevant Transcripts. We next sought to assess whether our inducible CITS editor could be deployed to edit

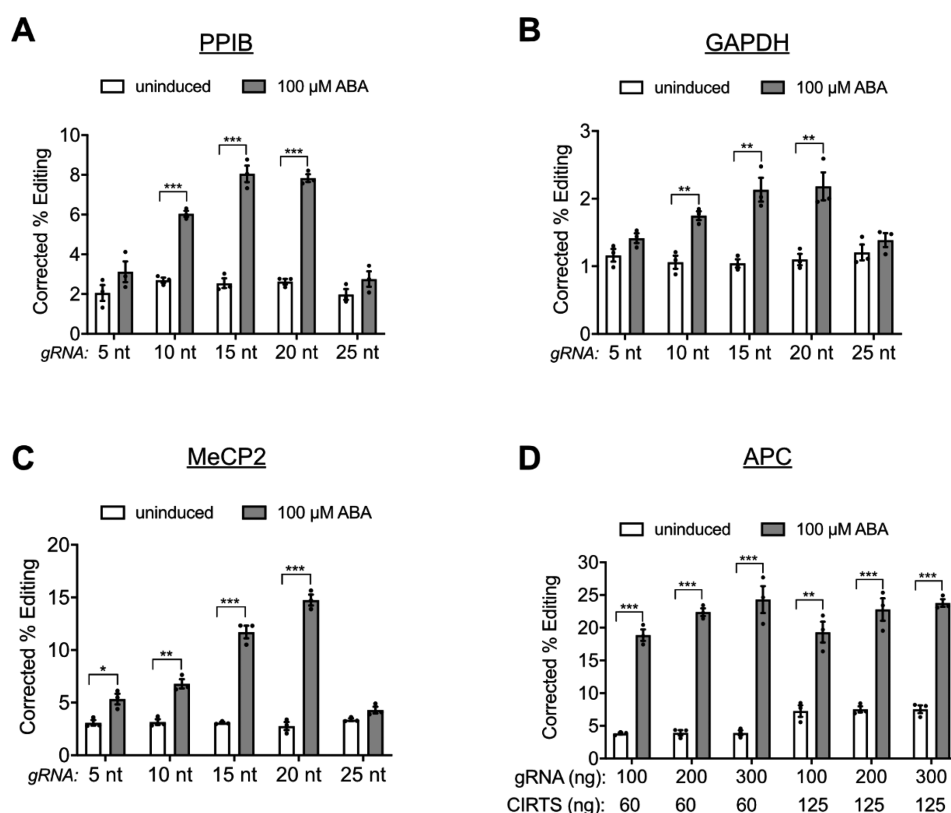


Figure 3. Inducible RNA editing on endogenous/disease targets. Inducible CIRTIS editor can be deployed to endogenous targets (A) PPIB and (B) GAPDH (both UAG sites within the ORF) or disease-relevant reporter transcripts (C) MeCP2 and (D) APC and shows ABA-dependent RNA editing. For all experiments, HEK293T cells were transfected with the CIRTIS biosensor vector and an on-target gRNA expression vector. The editing biosensor was induced 24 h after transfection, and RNA levels were quantified by RT-PCR-Sanger sequencing 48 h after induction. All values are mean $\pm$ SEM with $n = 3$ biological replicates. Student t test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

endogenous transcripts and disease-relevant mutations. To showcase the ability of the inducible CIRTIS editor to target endogenous transcripts and verify the programmable nature of the system, we chose two UAG editing sites within the open reading frame (ORF) of endogenous transcripts, GAPDH and PPIB, which have both been previously targeted by other RNA base editor systems.^{44,45} We tiled gRNAs along the endogenous transcripts and introduced the CIRTIS biosensor editor and each gRNA sequence to cells, induced with ABA or not, and then quantified the editing levels 48 h later. Indeed, we observed statistically significant, albeit low, levels of editing for both of the targeted transcripts, dependent on small molecule activation of the system (Figure 3A,B). As with our luciferase reporter (Figure S1A and S1B), we determined a mismatch 15–20 nt into the guiding sequence yielded the best editing efficiency.

After validating that we could target endogenous RNAs, we next sought to reverse known disease-causing mutations. We initially chose a G-to-A mutation, R106Q, in human Methyl CpG Binding Protein 2 (MeCP2), which has previously been targeted by other RNA base editor systems.^{46,47} Mutations in MeCP2 cause the neurological disorder Rett Syndrome with R106Q more commonly occurring than other mutations and resulting in a more severe form of Rett Syndrome. We cloned a MeCP2 isoform B reporter construct with a R106Q mutation fused to GFP and designed guides to tile the reporter transcript. We then delivered the CIRTIS biosensor, MeCP2 targeting gRNA, and the MeCP2 reporter. After inducing with ABA or not for 48 h, we used RT-PCR-Sanger sequencing to

analyze RNA editing. Our CIRTIS biosensor system can revert the R106Q mutation in a small molecule-dependent manner, and a 20 nt mismatch in the guiding sequence yielded the best editing efficiency (Figure 3C). In addition, we chose a stop codon-inducing mutation in adenomatous polyposis coli (APC) as a test case. APC is a tumor suppressor gene, with premature stop codons in APC present in 95% of familial adenomatous polyposis (FAP) patients.⁴⁸ To simulate a disease-causing mutation of APC, we searched NCBI's ClinVar database for disease-causing G-to-A mutations resulting in a premature stop codon and subsequently cloned an APC reporter construct containing a fraction of the APC gene with a W1262X (X = STOP) mutation. We utilized this reporter system to optimize editing efficiency by delivering varying concentrations of the CIRTIS biosensor and APC targeting gRNA, with a constant concentration of the APC stop codon-simulating reporter. After induction with ABA or not, we again assessed RNA editing using the RT-PCR-Sanger sequencing assay. We found that our CIRTIS biosensor system can efficiently revert the stop codon mutation in this simulated disease model and that increasing the ratio of gRNA:CIRTIS biosensor can improve editing efficiency by reducing editing in the absence of ABA (Figure 3D).

Modularity of the CIRTIS Biosensor for RNA Targeting. Encouraged by the ability to convert a CIRTIS editor into a biosensor for reporter and endogenous targets, we next aimed to assess the generality of the design to create small molecule controllable versions of other RNA effector systems. We swapped the effector domain from hADAR2(E488Q) to

three previously validated CIRT's effectors: the Pin nuclease domain to induce RNA degradation,⁴⁹ the m⁶A reader protein YTHDF1, which should induce translational activation,²⁵ and the m⁶A reader YTHDF2, which should induce RNA degradation.^{22,50,51} As expected, cells transfected with the Pin nuclease domain and the YTHDF2 reader showed small molecule-inducible RNA degradation (Figure 4A and Figure

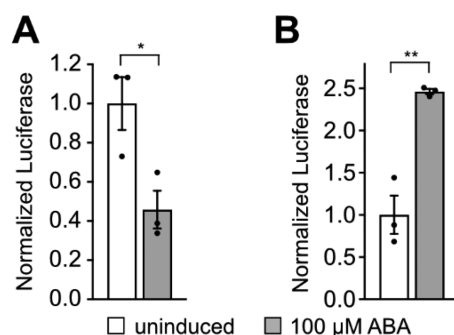


Figure 4. Versatility of the CIRT's biosensor. (A) The hADAR2-(E488Q) effector was swapped with previously validated CIRT's effector Pin nuclease and (B) YTHDF1. Cells transfected with the CIRT's biosensor vector and an on-target gRNA were subjected to a luciferase reporter assay 24 h after biosensor induction. We observed ABA-dependent RNA degradation via Pin nuclease and translation activation via YTHDF1. All values are mean $\pm$ SEM with $n = 3$ biological replicates. Student t test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

S3A). ABA induction of the YTHDF1 effector system resulted in the expected increase in luciferase readout due to activating translation on the target RNA (Figure 4B, Figure S3B). Taken together, these findings show that a versatile range of effectors can be used with the ABA-inducible CIRT's biosensor to both study RNA binding protein dynamics in cells and to control gene expression temporally.

In Vivo Editing Using a CIRT's Biosensor. Finally, we aimed to assess whether our CIRT's biosensor can be deployed *in vivo* using an animal model. As we had never delivered CIRT's into mice before, we first aimed to test whether the full-length (i.e., noninducible) CIRT's construct could function *in vivo* as an RNA base editor. We optimized CIRT's-hADAR2(E488Q) plasmids for delivery to the mouse liver by constructing a single plasmid encoding the CIRT's base editor system and gRNA targeting the luciferase reporter (Figure 5A) and a mouse reporter plasmid with the EF1 α promoter for robust, long-term expression of luciferase. To ensure the all-in-one system still functioned properly, we transfected HEK293T cells with the single CIRT's-gRNA plasmid and the cell or mouse luciferase plasmid and observed comparable editing to the original multivector system (Figure S4A). Based on this validation, we delivered the stop codon mutation-containing “dead” luciferase reporter by hydrodynamic tail vein injection to the mouse liver at 2 or 20 μ g to determine optimal reporter concentration. No significant signal was observed with lower DNA concentrations, and we therefore selected 2 μ g of reporter for future experiments (Figure S4B). We then tested full-length CIRT's-hADAR2(E488Q) delivered with the luciferase reporter by hydrodynamic tail vein injection. Delivery was optimized so the robust luciferase signal at 7 h postinjection was only observed in the presence of on-target gRNA (Figure 5B, Figure S5A,B). No significant editing was observed with a nontargeting gRNA and CIRT's-hADAR2-

(E488Q) as compared to mice receiving reporter alone. Collectively, these experiments show that the full-length CIRT's RNA base editor is functional *in vivo*, providing the first example of CIRT's activity in a live animal, and one of only a few examples of site-directed RNA base editing *in vivo*.⁴³

Since the full-length system induced detectable editing, we aimed to similarly optimize ABA-CIRT's-hADAR2(E488Q) delivery and establish ABA delivery conditions for small molecule-inducible editing. Again, we first engineered a “combined” vector, which expresses both protein components of the ABA-inducible base editor, as well as the gRNA, and validated the system still performed well in cell culture experiments (Figure 5C, Figures S6 and S7). Next, we deployed the system *in vivo*. ABA administration postplasmid delivery was based on previously determined gene expression patterns in the liver following hydrodynamic tail vein injection^{52,53} and ABA clearance rates.⁴⁰ We tested ABA injection intraperitoneally (i.p.) or intravenously (i.v.), 2 and 6 h after the plasmid expressing ABA-CIRT's-hADAR2(E488Q), and gRNA was delivered along with the mouse reporter plasmid. Significant editing of the luciferase mutation, as measured by the luciferase signal, was only observed in the presence of ABA and could be achieved with either ABA delivery method (Figure 5D, Figure S8). In the absence of ABA, no significant luminescence was observed over the reporter control conditions. Collectively, these results demonstrate that CIRT's approaches can be utilized for RNA editing of a reporter transcript of interest and small molecule-inducible editing *in vivo*.

DISCUSSION

Using the ABA chemical-inducible system, we developed a programmable and small-molecule-inducible RNA-targeting effector system, based on our previously engineered CIRT's technology. The key advance of this work lies in the added ability to both study and control a versatile set of RNA regulatory mechanisms in a small molecule-dependent manner. In this study, we achieved ABA-dependent RNA editing, RNA degradation, and translation initiation. However, the versatile nature of the CIRT's biosensor also provides the ability to study rapid RNA regulatory processes, such as mRNA splicing or translation, in complex systems such as in mouse models. In this proof-of-concept demonstration, we utilized well-characterized RNA modification inducers or readers in order to demonstrate that the system tolerates effector swaps and is likely a generalizable approach for targeted RNA manipulation. In addition to exchanging the effector domain, other orthogonal small molecule- or light-inducible heterodimerization domains, including the FRB/FKBP rapamycin system, the GID1/GAI gibberellin system, or the blue light-based CRY2/CIBN pairs,³⁷ could open up the possibility of studying temporal dynamics of orthogonal regulatory pathways simultaneously.

We acknowledge that our current biosensor system yields relatively low levels of RNA editing on endogenous transcripts. The low editing efficiency of the current biosensor system precludes direct usage for some biological studies, such as to studying RNA editing on many endogenous transcripts in cell lines or mouse models. We suspect that one likely reason for the observed modest editing efficiencies is the fact that endogenous transcripts are highly structured and bound by RNA binding proteins that can block accessibility for CIRT's. However, as we see almost no loss in activity for any of the

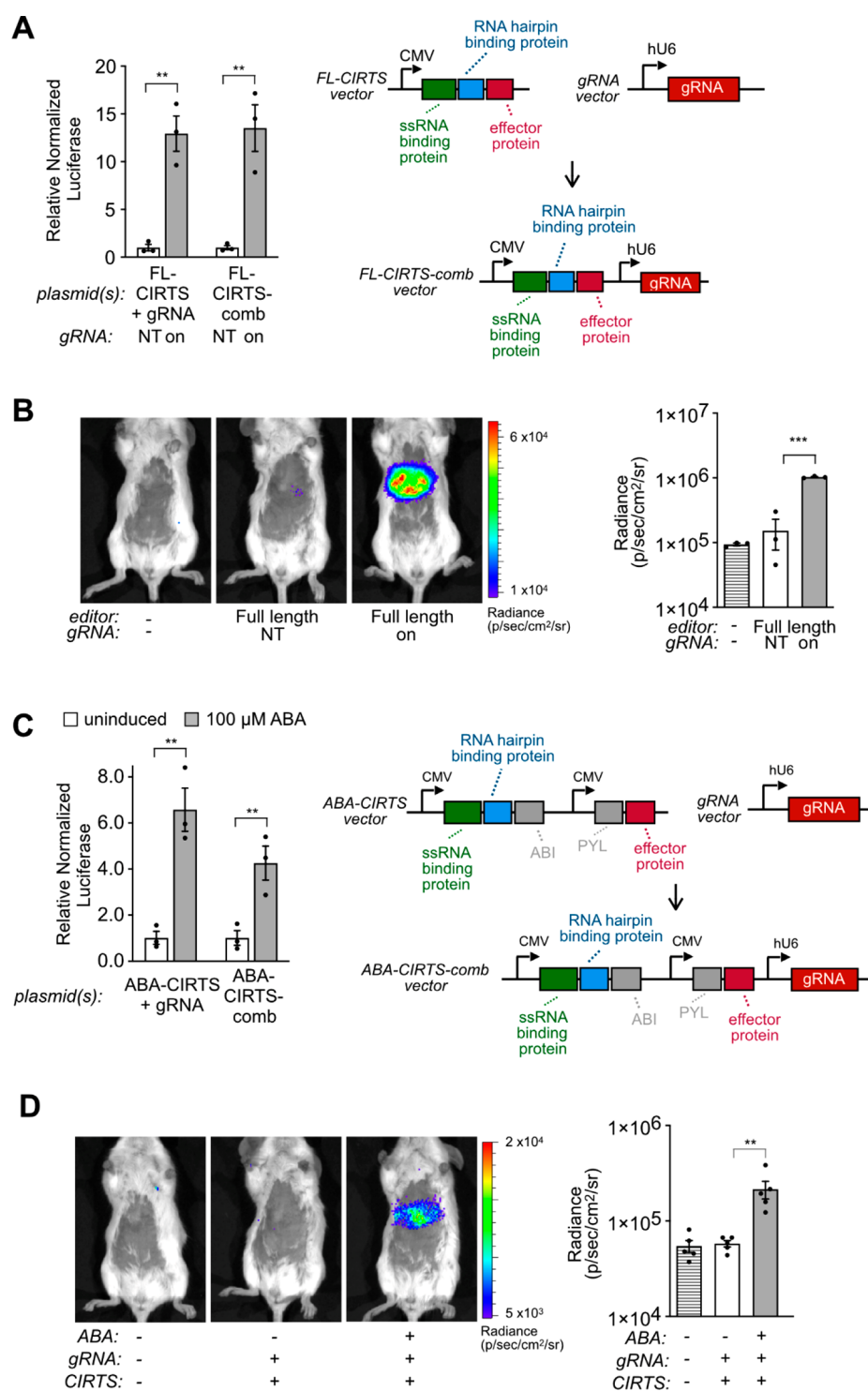


Figure 5. *In vivo* RNA editing with CIRTShADAR2(E488Q). (A) Cells transfected with a two-plasmid system for full-length CIRTShADAR2(E488Q) and gRNA were compared to cells transfected with a combined, single plasmid system. The biosensor was induced 24 h after transfection, and cells were subjected to a luciferase reporter assay 24 h after induction. (B) *In vivo* editing of a luciferase reporter with full-length CIRTShADAR2(E488Q). DNA encoding full-length CIRTShADAR2 with on-target or nontargeting gRNA was delivered with a luciferase reporter by hydrodynamic tail vein injection. Luciferin was administered i.p., and photon outputs were quantified. (C) Comparable to the experimental setup in A, cells transfected with a two-plasmid system for ABA-CIRTShADAR2(E488Q) and gRNA were compared to cells transfected with a combined, single plasmid system. (D) Small molecule inducible editing of a luciferase reporter with CIRTShADAR2(E488Q) biosensor. DNA encoding ABA-CIRTShADAR2(E488Q) was delivered with a luciferase reporter by hydrodynamic tail vein injection. Mice were imaged as in (b). Representative bioluminescence images are shown for B and D. All values are mean $\pm$ SEM with (A–C) $n = 3$ or (D) $n = 5$ biological replicates. Student t test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

other RNA effector proteins tested (Pin nuclease, YTHDF1, and YTHDF2), we also believe that this could be an editor-specific issue.

Like our system, λ N-ADAR and dCas13b-ADAR (REPAIR) editing systems rely on delivery of a protein component and a gRNA and yield average editing efficiencies of 10–20% and 3–30% on endogenous mammalian targets, respectively, when delivered by plasmid transfection.^{29,54} In addition, plasmid transfections of λ N-ADAR for the disease-relevant MeCP2 reporter have been utilized to repair R106Q with an average editing efficiency of 50%.⁴⁷ While biosensors of these systems have not been developed to enable direct comparisons to our small molecule-inducible CIRTs, we do observe relatively similar editing activity with full length CIRTs-hADAR2(E488Q) and REPAIR (Figure S9A). Additionally, in previous work on the development of site-specific editing technology, including SDREs and REPAIR,²⁹ targeting endogenous editing sites initially showed variable and often low editing efficiencies that required extensive optimization until therapeutically relevant editing levels were reached. We therefore believe that in future studies, the relative capacity of the CIRTs editing biosensor can likely be improved by protein engineering and directed evolution of the effector.

In general, we and others have found that both the gRNA structure as well as the landing site are crucial for performance. In this work, we were inspired by Montiel-Gonzalez et al.⁴² to adapt a two-hairpin gRNA structure that improved editing efficiency significantly (Figure 1D). In previous work with guided RNA editors, researchers were able to improve editing efficiency by varying the mismatch position within the gRNA.^{29,43} We believe that combining these approaches and testing different gRNA design strategies and landing sites for each specific endogenous transcript would allow for rapid optimization to adapt the CIRTs biosensor system for a broad range of applications. Depending on the application, alternative approaches of protein engineering to increase effects at the target site could include the use of the Sun-tag system to recruit more effects/targeting event⁵⁵ or the inclusion of additional hairpin binding components to increase the binding affinity.⁴² Lastly, experimental details such as the ratio of introduced protein and gRNA plasmids can effect on-target efficiencies for different applications. We observed that the published gRNA:editor ratio of site-directed RNA editors (SDRE)s greatly exceeds our current conditions (SDREs: 10:1⁴⁷ or 15:1,⁵⁴ Cas13-ADAR 2:1²⁹ vs CIRTs: 0.6). Adjusting these parameters was also helpful to optimize biosensor performance on a disease-relevant reporter target and will be further explored for endogenous transcripts in the future.

One additional concern with engineered protein-based editing systems is their selectivity. In previous studies developing site-specific RNA editing technologies, off-target effects as a result of hADAR2 overexpression have been reported.⁵⁶ As our system relies on overexpression of a hADAR2-containing protein construct, our system will have similar challenges with off-targets as other state-of-the-art programmable editing technologies. However, we did assess potential off-target effects of the CIRTs editor biosensor on the target luciferase transcript by comparing a no ABA with an ABA-treated sample using high-throughput sequencing (Figure S9B). We found that our target site was efficiently edited (21%) in the ABA-treated sample, while observing several off-target editing sites in both the no ABA and ABA samples. We

observed more nontargeted edits in the ABA-treated sample, suggesting that the hADAR2(E488Q) enzyme is brought to proximity of the luciferase transcript in the sample. However, all observed nontargeted edits were observed at lower editing efficiencies than the on-target site (Figure S9B).

More broadly, this programmable, small molecule-controllable RNA effector design provides a generalizable approach to study the RNA regulatory dynamics of an ever-growing list of known RNA regulatory proteins in live cells. Several recent advances in understanding the regulatory role of individual chemical modifications, such as m⁶A, have relied on programmable site-specific targeting technology such as RNA-targeting Cas9 (RCas9) or Cas13.^{57–59} For example, a programmable dCas13b-FTO protein was instrumental in demonstrating the role m⁶A modifications on specific chromosome-associated regulatory RNAs play in transcription.⁶⁰ Despite these advances in understanding regulatory roles of m⁶A on specific transcripts, the temporal component of their regulation has yet to be elucidated. We imagine CIRTs biosensors with effectors such as METTL3 to install or FTO demethylase to remove such modifications could provide a versatile tool to study, tune, and understand transcription in a temporally controlled manner.

We further demonstrate that RNA editing with full length CIRTs-hADAR2(E488Q) or the ABA editing biosensor can be deployed in mice—providing the first *in vivo* demonstration of CIRTs. We observed similar fold-changes in luminescence output in our cellular and *in vivo* experiments, though *in vivo* normalizations were compared to a mice receiving reporter, only as multicomponent imaging of both Firefly and Renilla luciferase is very challenging on these time scales.^{61,62} While careful tuning of both plasmid and ABA delivery is necessary in the hydrodynamic tail vein injection-based gene delivery model used here, it ultimately affords temporal control on the hour-time scale *in vivo*. While editing efficiency of the current biosensor is likely too low for direct use on endogenous targets, further optimization of plasmid and ABA delivery could likely afford improvements in editing efficiency and would be necessary if editing on different time scales is needed. For both the i.p. and i.v. injections of ABA in these experiments, we administered the highest possible concentration of a small molecule based on ABA solubility. Our delivery strategy was based on previously published stability and detections assays with ABA in mice.⁴⁰ While we did not optimize conditions for ABA concentration, it is possible that less ABA could be administered to obtain significant editing and will likely need to be tuned for other CIRTs biosensor effector domains.

We are also currently investigating additional delivery methods of the CIRTs system for improved RNA targeting and to move toward more clinically relevant *in vivo* targets. Based on our previous work and the total size of the single plasmid optimized CIRTs-hADAR2(E488Q) (3.1 kb, Figure 5A) or ABA-CIRTs-hADAR2(E488Q) (4.7 kb, Figure 5C), we anticipate either could be efficiently packaged and delivered with adenovirus-associated virus. We are also pursuing nonviral delivery systems, including liposomes and lipid nanoparticles. These additional delivery approaches are likely to be broadly applicable to RNA targeting of different disease states and physiological processes with CIRTs. Collectively, the validation of the inducible CIRTs technology demonstrates the feasibility of an RNA-targeting, inducible biosensor and

lays the foundation for temporally regulated studies of RNA regulation in mammalian systems.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscentsci.0c00537>.

Experimental details on ABA CIRTs biosensor design, mammalian cell RNA targeting assays, and *in vivo* imaging (PDF)

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Author Contributions

[§]S.R. and K.A.J. contributed equally to this work.

Notes

The authors declare the following competing financial interest(s): B.C.D. and S.R. have filed a provisional patent on CIRTs.

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