RNA regulation is critical for gene expression control, but tools to temporally manipulate RNA regulatory mechanisms are lacking. Here, we present small molecule-inducible RNA-targeting effectors based on our previously developed CRISPR/Cas-inspired RNA targeting system (CIRTS), which can trigger RNA editing, degradation, or translation on target transcripts. We go on to show the inducible CIRTS editor can be deployed for RNA base editing in vivo, providing a new set of tools to probe RNA regulatory dynamics and control gene expression.
**Small molecule-inducible RNA-targeting systems for temporal control of RNA regulation *in vivo***

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RNA regulation is critical for gene expression control, but tools to temporally manipulate RNA regulatory mechanisms are lacking. Here, we present small molecule-inducible RNA-targeting effectors based on our previously developed CRISPR/Cas-inspired RNA targeting system (CIRTS), which can trigger RNA editing, degradation, or translation on target transcripts. We go on to show the inducible CIRTS editor can be deployed for RNA base editing *in vivo*, providing a new set of tools to probe RNA regulatory dynamics and control gene expression.

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. Apart from canonical RNA processing including capping, splicing, polyadenylation, and trafficking, RNA sequences can be edited or chemically modified\(^1\). Adenosine-to-inosine (A-to-I) editing is a common post-transcriptional modification\(^2\). The hADAR family of proteins deaminate A in double-stranded RNA (dsRNA) at the site of a C-A mismatch. The deamination product inosine mimics guanosine (G) in cells by base-pairing with cytosine (C), allowing for both coding amino acid and splicing changes\(^3\). In addition to RNA editing, RNA bases can be chemically modified. \(N^6-\)
methyladenosine (m$^6$A) is the most prevalent internal RNA modification in mammalian systems. m$^6$A is dynamically installed and erased by writer and eraser enzymes, respectively and is linked to cellular functions by recognition and binding of m$^6$A reader proteins. Chemical RNA modifications influence RNA stability, splicing, export, and translation efficiency and have been linked to various cancers and viral infections. With increasing numbers of known 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 the dynamics of biological processes as well as therapeutic applications. For example, Cas13 proteins of the CRISPR-Cas system have been developed to deliver a variety of effector proteins, including RNA editors, and translational activators to RNA transcripts and sites of interest. We recently developed CIRTS as a smaller, human-derived delivery moiety for RNA effectors. CIRTS relies on guide RNA (gRNA) complementarity to bind a target of interest and deliver a tethered cargo protein. While these tools can deliver RNA regulatory proteins to a transcript and site of interest, they offer no temporal control to study effector dynamics. Even though inducible DNA-targeting technologies, primarily based on the Cas9 protein family, have provided valuable tools to probe genetic regulation, comparable systems for transcriptomic regulation are 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 editing, degradation, and translation.

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. 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. We fused the targeting component of CIRTS, a single-stranded RNA binding protein and hairpin binding protein, to ABI (CIRTS-ABI).
and the RNA effector domain to PYL (Figure 1A). The targeting CIRTS component binds to a
gRNA and engages the target RNA by base pair complementarity. Addition of ABA recruits the
effector domain to the targeting moiety of CIRTS to elicit the desired function on the targeted RNA
transcript.
       We validated the ABA biosensor in 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
editing (Figure S1A). After validating our system by transfecting cells with targeting CIRTS-ABI,
a gRNA targeting the mutated site, and PYL fused to hADAR2(E488Q), we constructed a
combined CIRTS-ABI and PYL-hADAR(E488Q) vector to reduce the number of transfected
plasmids. We then screened gRNAs to find the ideal mismatch position along our gRNA (Figure
S1B and S1C) and determined a mismatch 15nt into the guiding sequence yielded the best editing
efficiency.
       In previous studies, it was reported that editing systems are more efficient when the gRNA
contained two hairpin sequences\textsuperscript{19}. When a second TAR hairpin was added to the gRNA design,
we observed a significant increase in editing efficiency (Figure 1B). To control for gRNA-
dependent editing due to endogenous ADAR, we performed control experiments, delivering each
individual component of our system. We observed some low levels of background editing when
only the gRNA was delivered (Figure 1C and Figure S1D), but only saw robust editing when we
transfected cells with the ABA-CIRTS biosensor and gRNA, and induced the system with ABA
(Figure 1C). We compared the biosensor CIRTS editor to the full-length CIRTS editor and found
that conversion of CIRTS into a biosensor only results in a two-fold loss of efficacy (Figure S1E).
       After validating our inducible CIRTS biosensor, we next assayed the temporal dynamics
of the inducible editing response. We conducted an ABA time course experiment and measured
the resulting RNA editing levels for three days. Editing efficiency was monitored by both a cell-
based luciferase stop codon reversion assay and by quantifying RNA edits directly using reverse
transcription (RT)-polymerase chain reaction (PCR) followed by Sanger sequencing and a
correction for fluorophore and PCR biases with a standard curve (Figure S1F). Both G content at the mutation site and luciferase readout increase steadily over the course of 72 h of ABA-induced editing via our CIRTS biosensor (Figure 1D and S1G).

Encouraged by the ability to convert a CIRTS editor into a biosensor, we swapped the effector domain from ADAR to three previously validated CIRTS effectors: the Pin nuclease domain to induce RNA degradation\textsuperscript{20}, the m\textsuperscript{6}A reader protein YTHDF1 to induce translational activation\textsuperscript{10}, and the m\textsuperscript{6}A reader YTHDF2 that induces RNA degradation\textsuperscript{7}. As expected, cells transfected with the Pin nuclease domain and the YTHDF2 reader showed small molecule-inducible RNA degradation (Figure 1E and S1H). ABA induction of the YTHDF1 effector system resulted in the expected increase in luciferase readout due to activating translation on the target RNA (Figure 1E). Taken together, these findings show that a versatile range of effectors can be used with the ABA-inducible CIRTS biosensor to both study RNA binding protein dynamics in cells and control gene expression temporally.

We then sought to assess whether our inducible CIRTS editor could be deployed to edit disease-relevant mutations or endogenous transcripts. To showcase the ability of the inducible CIRTS editor to reverse a known disease-causing mutation, we chose a stop codon-inducing mutation in adenomatous polyposis coli (APC) as our test case. APC is a tumor suppressor gene, with premature stop codons in APC present in 95% of familial adenomatous polyposis (FAP) patients\textsuperscript{21}. We again delivered the CIRTS biosensor, APC targeting gRNA, as well as an APC stop codon-simulating reporter, and assessed RNA editing using the RT-qPCR-Sanger sequencing assay. We found that our CIRTS biosensor system can efficiently revert the stop codon mutation in this simulated disease model (Figure 2A). We furthermore chose two endogenous transcripts, GAPDH and PPIB, to verify the programmable nature of the system. We introduced the CIRTS biosensor editor and a gRNA sequence to cells and quantified editing levels 48 h after induction of the system. We observed significant, albeit low levels, of editing for both of the targeted transcripts (Figure 2A).
Finally, we aimed to assess whether our CIRTS biosensor can be deployed *in vivo* using a mouse model. We optimized the plasmids for delivery to the mouse liver by constructing a single plasmid encoding the CIRTS system and gRNA and a mouse reporter plasmid with the EF1a promoter for robust, long-term expression of luciferase. To ensure the system still functioned properly, we transfected HEK293T cells with the single CIRTS-gRNA plasmid and the cell or mouse luciferase plasmid and observed comparable editing (Figure S2A-B). Based on this validation, we delivered the luciferase reporter by hydrodynamic tail vein injection to the mouse liver at 2 or 20 µg to determine optimal reporter concentration. No significant signal was observed with lower DNA concentrations and was selected for future experiments (Figure S2C). For *in vivo* editing, we first tested full length CIRTS-hADAR delivered with the luciferase reporter by hydrodynamic tail vein injection. Delivery was optimized so robust luciferase signal at 7 h post injection was only observed in the presence of on-target gRNA (Figure 2B, Figure S3A-B). No significant editing was observed with a non-targeting gRNA and CIRTS-hADAR as compared to mice receiving reporter alone.

Since the full length system induced robust editing, we aimed to similarly optimize ABA-CIRTS-hADAR delivery and establish ABA delivery conditions for small molecule-inducible editing (Figure S4A-B). ABA administration post plasmid delivery was based on previously determined gene expression patterns in the liver following hydrodynamic tail vein injection\textsuperscript{22-23} and ABA clearance rates\textsuperscript{17}. ABA was injected intraperitoneally (i.p.) or intravenously (i.v.) 2 h and 6 h after plasmid expressing ABA-CIRTS-hADAR and gRNA was delivered with the mouse reporter plasmid. Significant editing of the luciferase mutation was only observed in the presence of ABA and could be achieved with either ABA delivery method (Figure 2C, Figure S4C-D). In the absence of ABA, no significant luminescence was observed over the reporter control conditions. Collectively, these results demonstrate that CIRTS approaches can be utilized for RNA editing of a transcript of interest and small molecule-inducible editing *in vivo*. 
Using the ABA chemical-inducible system, we developed the first inducible RNA targeting system based on our engineered CIRTS technology. In this study, we achieved ABA-dependent RNA editing, RNA degradation, and translation initiation. 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\textsuperscript{16}, 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. As we see almost no loss in activity for any of the other RNA effector proteins tested (Pin nuclease, YTHDF1, and YTHDF2), we believe that this could be an editor-specific issue. In future studies, the relative capacity of the CIRTS editing biosensor can likely be improved by protein engineering such as directed evolution of the effector and a linker or orientation screen to improve effector-mutation site alignment. Additionally, there are a range of other RNA regulatory proteins that could be fused to the CIRTS biosensor to study RNA regulation dynamics in live cells. We further demonstrate that RNA editing with full length CIRTS-hADAR or the ABA editing biosensor can be deployed in mice – providing the first \textit{in vivo} application of CIRTS. While careful tuning of both plasmid and ABA delivery are necessary in the liver model used here, it ultimately affords temporal control on the hour-timescale \textit{in vivo}. Further optimization of plasmid and ABA delivery could likely afford improvements in editing efficiency and would be necessary if editing on different timescales is needed. We are also currently investigating additional delivery methods of the CIRTS system to move towards more clinically relevant \textit{in vivo} targets. 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.
Figure 1 Development of a CIRTS biosensor.
(A) Schematic of the small molecule-inducible CIRTS biosensor. (B) gRNA comparison of the original 1 TAR gRNA with a 2 TAR gRNA. (C) Cells transfected with each individual component 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. (D) Quantified editing percentage by RT-Sanger sequencing in a time course after addition of ABA. (E) The hADAR effector was swapped with previously validated CIRTS effector Pin nuclease and YTHDF1. We observed ABA-dependent RNA degradation via Pin nuclease and translation activation via YTHDF1. All values are mean ± SEM with n = 3 biological replicates. Student t-test: *P < 0.05, **P <0.01, ***P <0.001
Figure 2 Inducible RNA editing on endogenous/disease targets and in vivo editing.

(A) The inducible CIRTS-editor can be deployed to disease-relevant reporter transcripts (APC) or endogenous targets (GADPH and PPIB) and shows ABA-dependent RNA editing.

(B) In vivo editing of a luciferase reporter with full length CIRTS-hADAR. DNA encoding full length CIRTS-hADAR with on-target or non-targeting gRNA was delivered with luciferase reporter by hydrodynamic tail vein injection. Luciferin was administered i.p. and photon outputs were quantified. (C) Small molecule inducible editing of a luciferase reporter with CIRTS biosensor. DNA encoding ABA-CIRTS-hADAR was delivered with luciferase reporter by hydrodynamic tail vein injection. Mice were imaged as in (b). Representative bioluminescence images are shown for B-C. All values are mean ± SEM with (A-B) n = 3 or (C) n = 5 biological replicates. Student t-test: *P < 0.05, **P < 0.01, ***P < 0.001
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Competing Interests

B.C.D. and S.R. have filed a provisional patent on CIRTS.
METHODS

Cloning. All plasmids were cloned using Gibson Assembly and sequenced by the University of Chicago Comprehensive Cancer Center DNA Sequencing and Genotyping Facility. PCR fragments for Gibson Assembly were generated using Q5 DNA Polymerase (NEB). All plasmids used in this study are listed in Table S1 with links to their vector maps and are available upon request. Key plasmids will be made available through Addgene.

Mammalian Cell Culture and Transfection. For cell culture assays, HEK293T cells (ATCC) were used. Cells were maintained in DMEM (L-glutamine, high glucose, sodium pyruvate, phenol red; Corning) media supplemented with 10% fetal bovine serum (FBS; Gemini Benchmark), and 1x penicillin/streptomycin (P/S; Gibco/Life Technologies). For transfections, P/S was omitted from the media. All transfections were conducted using lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations.

Induction Luciferase Assays. For ABA induction experiments, HEK293T cells were transfected with 12.5 ng reporter plasmid, 150 ng indicated CIRTS biosensor vector, and 100 ng gRNA expression vector. Approximately 16h before transfection, cells were plated on 96-well plates and grown to 70-80% confluency overnight. The next day, cells were transfected with a total of 20 µL Opti-MEM I Reduced Serum Medium (ThermoFisher Scientific): 10 µL Opti-MEM containing 0.5 µL lipofectamine 2000 per well and 10 µL Opti-MEM containing the plasmid DNA. Diluted lipofectamine 2000 and DNA were combined and incubated for 15 min before slowly adding them to cells. After 24h, the ABA biosensor was induced with 100 µM abscisic acid (ABA, ThermoFisher) and incubated for an additional 24h before luciferase readout on a Biotek Synergy plate reader. Both firefly and Renilla luciferase readouts were measured as previously described (Jove paper Baker & Boyce). First, 40 µL growth media was removed from every well. Then, 40
µL of firefly assay buffer (Triton Lysis Buffer (50 mM Tris, pH 7.0, 75 mM NaCl, 3 mM MgCl₂, 0.25% Triton X-100) containing 5 mM DTT, 0.2 mM coenzyme A, 0.15 mM ATP, and 1.4 mg/mL D-luciferin) was added to lyse the cells and to provide the first substrate for firefly luciferase. After 10 min incubation, the firefly read was taken and 40 µL Renilla assay buffer (45 mM EDTA, 30 mM sodium pyrophosphate, 1.4 M NaCl, 0.02 mM PTC124, 0.003 mM coelentrazine h (CTZ-h)) was added to stop firefly luciferase activity and provide the substrate for Renilla luciferase. The Renilla read was taken immediately after addition of the buffer. All experiments were conducted in at least biological triplicates. Firefly luciferase luminescence levels were normalized to the corresponding Renilla luminescence levels to generate the normalized change in protein levels from the target firefly luciferase gene.

**Editing efficiency quantification.** To quantify the editing efficiency of CIRTS in mammalian cells, HEK 293T cells were transfected as described above. Cells were plated to be at 80% confluency and were transfected with 150 ng CIRTS expression vector and 100 ng luciferase reporter-targeting gRNA or 200 ng of gRNA when targeting endogenous transcripts or disease-simulated reporters. After 24h, the ABA biosensor CIRTS was induced with 100 µM ABA and incubated for an additional 24h (or 48h for endogenous/disease targets) until further processing. Total RNA was harvested using the RNeasy Mini Kit (Qiagen) when isolated from cells. After RNA purification, the resulting RNA was reverse transcribed with a target specific primer (Table S4) and the PrimeScript RT Reagent Kit (TaKaRa). The resulting cDNA product was PCR-amplified with Q5 DNA polymerase (NEB) using target-specific primers and sent for Sanger sequencing at the University of Chicago Comprehensive Cancer Center DNA Sequencing and Genotyping Facility. Editing efficiency was calculated by the ratio of peak heights at the target site (G/(A+G)) as previously reported (Ref in vivo editing ADAR Nat methods). A Sanger sequencing standard curve was used to correct editing efficiencies for sequencing fluorophore and PCR biases (Figure SX).
**In vivo delivery of CIRTS and imaging.** BALB/C mice (Charles River Laboratories) aged 5-6 weeks were injected with plasmids encoding the luciferase reporter (2-20 µg), full length CIRTS-hADAR2 (2-20 µg) or ABA-CIRTS-hADAR2 (2-20 µg) using TransIT-EE Hydrodynamic Delivery Solution (Mirus Bio) according to the manufacturer’s recommendations. Fur on the abdomen of the mice was removed 24-72 h prior to injection using a chemical depilatory cream. For ABA-CIRTS-hADAR2, animals received an i.p. injection of ABA (100 mg/kg, 200 µL per mouse, 2.5% DMSO) or i.v. injection of ABA (5 mM, 100 µL per mouse, 3% ethanol) 2 h and 6 h after plasmid delivery. At 7 h post plasmid delivery, animals received an i.p. injection of D-luciferin (100 mM, 100 µL per mouse, PBS pH 7.4). Mice were anesthetized (2% isoflurane) and placed on the warmed (37 °C) stage of an IVIS 200 Imaging System (Xenogen) and imaged with a CCD camera chilled to -90 °C at the University of Chicago Optical Imaging Core Facility. Exposure times were 1 minute with data binning levels set to medium. Regions of interest were selected for quantification and total flux values were analyzed using Living Image software. All animal experiments were performed following the protocols approved by the University of Chicago Institutional Animal Care and Use Committee.
