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A cytosine deaminase for programmable single-base RNA editing

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Programmable RNA editing enables reversible recoding of RNA information for research and disease treatment. Previously, we developed a programmable A to I RNA editing approach by fusing catalytically inactivated RNA-targeting CRISPR-Cas13 (dCas13) with the adenine deaminase domain of ADAR2. Here, we report a C to U RNA editor, referred to as RNA Editing for Specific C to U Exchange (RESCUE), by directly evolving ADAR2 into a cytidine deaminase. RESCUE doubles the number of pathogenic mutations targetable by RNA editing and enables modulation of phospho-signaling-relevant residues. We apply RESCUE to drive β -catenin activation and cellular growth. Furthermore, RESCUE retains A to I editing activity, enabling multiplexed C to U and A to I editing through the use of tailored guide RNAs.

We previously developed a RNA base editing technology called REPAIR (RNA editing for programmable A to I (G) replacement), which uses the RNA targeting CRISPR effector Cas13 (1–6) to direct the catalytic domain of ADAR2 to specific RNA transcripts to achieve adenine to inosine conversion with single-base precision (7). However, REPAIR, along with a number of other RNA editing technologies (8–15), only allow for A-to-I conversions. Technologies for precise RNA editing of cytidine to uridine would greatly expand the range of addressable disease mutations and protein modifications (fig. S1A).

Although natural enzymes capable of catalyzing C to U conversion have been harnessed for DNA base editing (16, 17), they only operate on single stranded substrates (18), exhibit off-targets (19–21), and deaminate multiple bases within a window. Therefore, we took a synthetic approach to evolve the adenine deaminase domain of ADAR2 (ADAR2dd), which naturally acts on double-stranded RNA substrates and preferentially deaminates a target adenine mispaired with a cytidine, into a cytidine deaminase. We fused this evolved cytidine deaminase to dCas13 to develop programmable RNA Editing for Specific C to U Exchange (RESCUE) in mammalian cells (fig. S1B), which we used to activate β -catenin and modulate cell growth. Lastly, we improved the specificity of RESCUE more than 10-fold via rational mutagenesis, generating a highly specific and precise C to U RNA editing tool.

Comparison of the *E. coli* cytidine deaminase and the human ADAR2dd showed remarkable structural homology between their catalytic cores (22), suggesting the possibility of

evolving ADAR2dd into a cytidine deaminase (fig. S1B). We selected residues of ADAR2dd contacting the RNA substrate (23) for three rounds of rational mutagenesis on an ADAR2dd fused to the catalytically inactive Cas13b ortholog from *Riemerella anatipestifer* (dRanCas13b), yielding RESCUE round 3 (RESCUEr3), with 15% editing activity (Fig. 1, A and B, and figs. S2 and S3). We then began directed evolution across ADAR2dd to identify additional candidate mutations that increase the activity of RESCUE in yeast (see supplementary methods and table S1). Sixteen rounds of evolution, culminating with the final construct RESCUEr16 (hereafter referred to as just RESCUE), resulted in increased cytidine deamination activity across all target combinations of neighboring 5' and 3' bases (Fig. 1C and figs. S4 to S7). We additionally characterized guide features necessary for robust activity, finding that RESCUE is optimally active with C or U base-flips across the target base using a 30-nt guide (Fig. 1C and figs. S8 to S9). Moreover, as dRanCas13b and the catalytically inactive Cas13b ortholog from *Prevotella sp. P5-125* (dPspCas13b) were equivalent, the final RESCUE construct used dRanCas13b (fig. S10).

The 16 mutations in RESCUE are distributed throughout the structure of ADAR2dd (fig. S11A), indicating both direct interactions of the evolved residues with the RNA target within the catalytic pocket as well as indirect effects (fig. S11B). These mutations enable fitting of either adenosine or cytidine, as RESCUE is capable of both adenosine and cytidine deamination (fig. S12). We evaluated the role of each mutant by individually adding them to REPAIR or removing

them from RESCUE. (fig. S13). We found that mutations in the catalytic core (V351G, K350I) and contacting the RNA target (S486A, S495N) were integral to RESCUE activity, while others had minor effects. Biochemical characterization of RESCUE mutations on purified ADAR2dd showed no activity on dsDNA, ssDNA, or DNA-RNA heteroduplexes, with the evolved mutations improving the kinetics of C to U editing on dsRNA substrates in vitro (fig. S14). We also found that ADAR2 or alternative RNA editing platforms without Cas13 (8, 9, 11, 13, 24) with introduced RESCUE mutations had markedly reduced editing compared to Cas13b-based RESCUE (Fig. 1D and figs. S15 to S18).

We next evaluated the efficiency of RESCUE on endogenous transcripts in HEK293FT cells via bulk sequencing of cell populations. We tested a variety of guide designs across 24 different sites across nine genes as well as on 24 synthetic disease-relevant mutation targets from ClinVar and found editing rates up to 42% (Fig. 1E, figs. S19 to S22, and table S2). Across the guides tested (tables S3 to S5), we found multiple guide design rules, most notably related to features of the motif (5' U or A preferred) and guide mismatch position (see supplementary materials and methods).

We next applied RESCUE to alter activation of the STAT and Wnt/ β -catenin pathways via modulation of key phosphorylation residues, which inhibits ubiquitination and degradation (25) (Fig. 2, A and B, and fig. S23). We tested a panel of guides targeting the β -catenin transcript (*CTNNB1*) at known phosphorylation residues and observed editing levels between 5% and 28% (Fig. 2C), resulting in up to 5-fold activation of Wnt/ β -catenin signaling (Fig. 2D) and increased cell growth in HEK293FT (Fig. 2, E and F) and human umbilical vein endothelial cells (HUVECs) (fig. S24). As therapeutic applications with RESCUE will require shorter constructs for viral delivery, we also evaluated RESCUE activity with C-terminal truncations of dRanCas13b and found either similar or improved deaminase activity (fig. S25).

Since RESCUE retains adenosine deaminase activity (fig. S12), the native pre-crRNA processing activity of Cas13b (4) enables multiplexed adenine and cytosine deamination. By delivering RESCUE along with a pre-crRNA targeting an adenine and a cytosine in the *CTNNB1* transcript (Fig. 3A), we found that RESCUE could edit both targeted residues S33F and T41A at rates of ~15% and 5%, respectively (Fig. 3B). However, in these experiments, as well as single-plex assays, we found A to I off-targets near the targeted cytosine (figs. S26 and S27). To eliminate these off-targets, we introduced unfavorable guanine mismatches in the guide across from off-target adenosines (Fig. 3C), significantly reducing off-target editing while minimally disrupting the on-target editing (Fig. 3D).

Because of off-targets observed near the target site, we profiled off-targets with whole-transcriptome RNA-

sequencing, finding that while RESCUE had ~80% C to U editing on the *Gluc* transcript (Fig. 4A), it had 188 C to U off-targets and 1,695 A to I off-targets, comparable to A to I off-targeting with REPAIRv1 (7) (Fig. 4, A and B). To improve the specificity of RESCUE we performed rational mutagenesis of ADAR2dd at residues interacting with the RNA target (Fig. 4C), resulting in improved specificity RESCUE mutants (Fig. 4, D to G). The top specificity mutant, S375A on RESCUE (hereafter referred to as RESCUE-S), maintained ~76% on-target C to U editing (Fig. 4E), but only had 103 C to U off-targets and 139 A to I off-targets (Fig. 4, E to G), with reduced missense mutations and differentially-regulated transcripts (figs. S28 to S31). We also found that RESCUE-S retained similar efficiency as RESCUE at endogenous sites with higher specificity (figs. S32 to S34).

RESCUE is a programmable base editing tool capable of precise cytidine to uridine conversion in RNA. Using directed evolution, we demonstrate that adenosine deaminases can be relaxed to accept other bases, resulting in a novel cytidine deamination mechanism that can edit dsRNA. The larger targetable amino acid codon space of RESCUE enables modulation of more post-translational modifications, such as phosphorylation, glycosylation, and methylation, as well as expanded targeting of common catalytic residues, disease mutations, and protective alleles, such as ApoE2 (figs. S1 and S35). Overall, RESCUE extends the RNA targeting toolkit with new base editing functionality, allowing for expanded modeling and potential treatment of genetic diseases.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S35

Tables S1 to S8

References (26–36)

Structure S1

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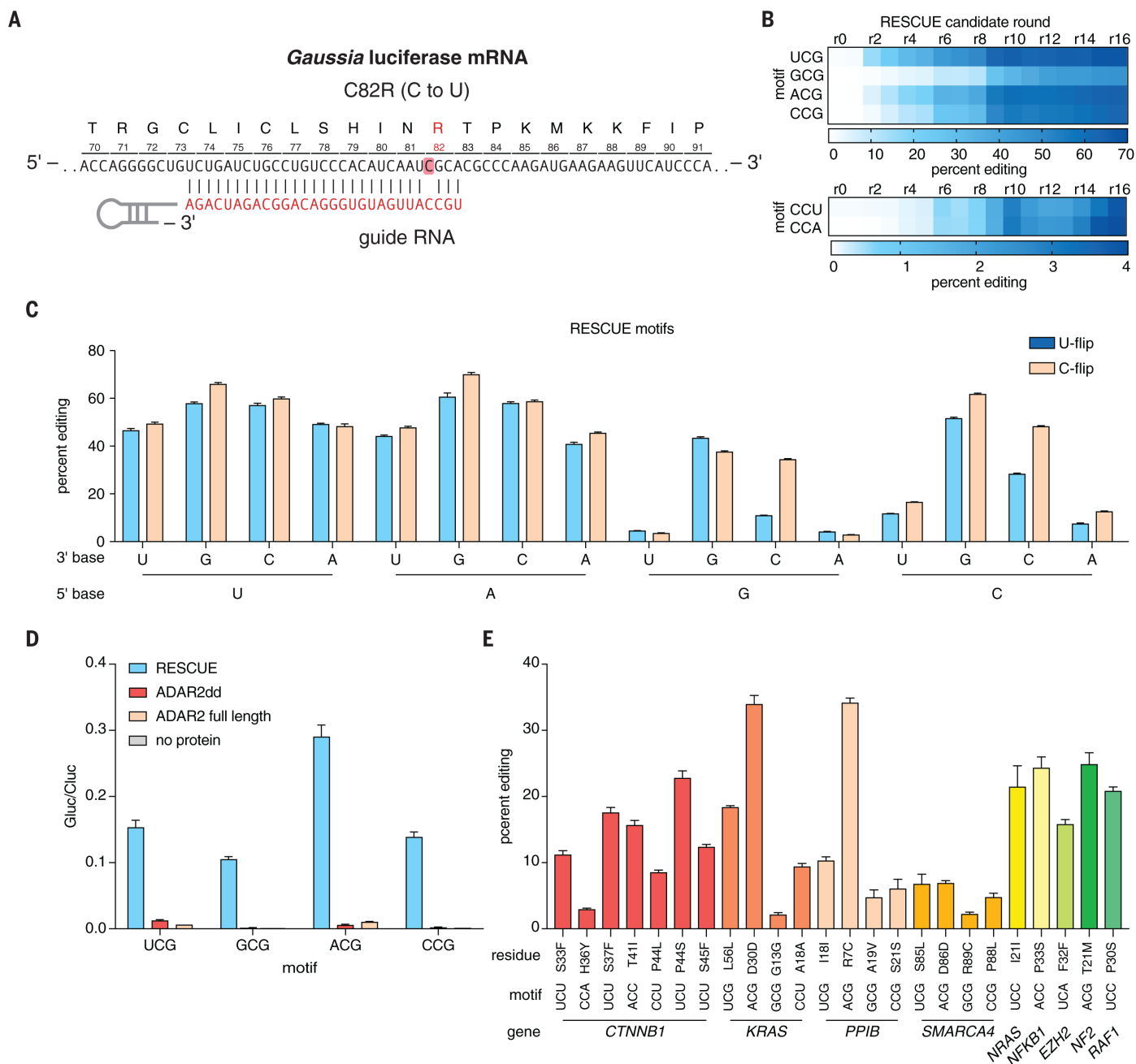


Fig. 1. Evolution of an ADAR2 deaminase domain for cytidine deamination. (A) Schematic of RNA targeting of the catalytic residue mutant (C82R) of *Gaussia* luciferase reporter transcript. (B) Heatmap depicting the percent editing levels of RESCUEr0-r16 on cytidines flanked by varying bases on the *Gluc* transcript. More favorable editing motifs are shown at the top, while less favorable motifs (5'C) are shown at the bottom. (C) Editing activity of RESCUE on all possible 16 cytidine flanking bases motifs on the *Gluc* transcript with U-flip or C-flip guides. (D) Activity comparison between RESCUE, ADAR2dd without Cas13, full-length ADAR2 without Cas13, or no protein. (E) Editing efficiency of RESCUE on a panel of endogenous genes covering multiple motifs. The best guide for each site is shown with the entire panel of guides displayed in fig. S19.

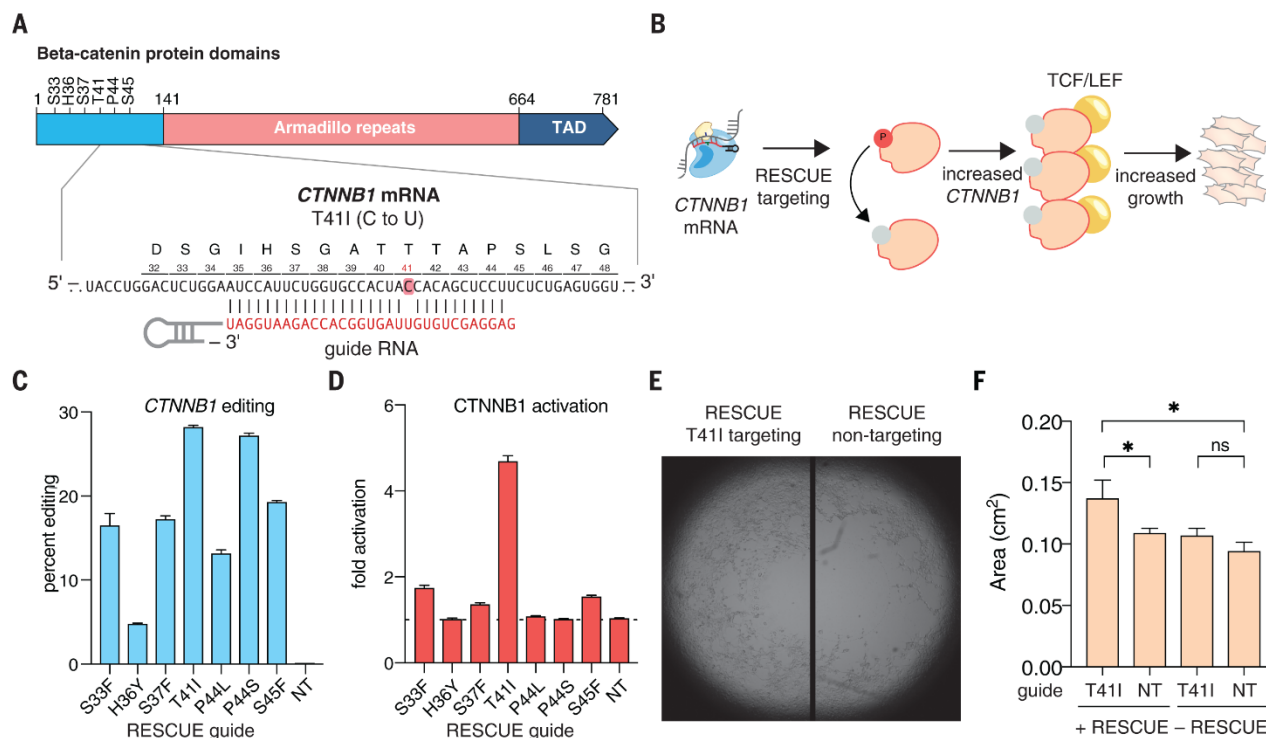


Fig. 2. Phenotypic outcomes of RESCUE on cell growth and signaling. (A) Schematic of β -catenin domains and RESCUE targeting guide. (B) Schematic of β -catenin activation and cell growth via RESCUE editing. (C) Percent editing by RESCUE at relevant positions in the *CTNNB1* transcript. (D) Activation of Wnt/ β -catenin signaling by RNA editing as measured by β -catenin-driven (TCF/LEF) luciferase expression. (E) Representative microscopy images of RESCUE *CTNNB1* targeting and non-targeting guides in HEK293FT cells. (F) Quantitation of cellular growth due to activation of *CTNNB1* signaling by RNA editing in HEK293FT cells.

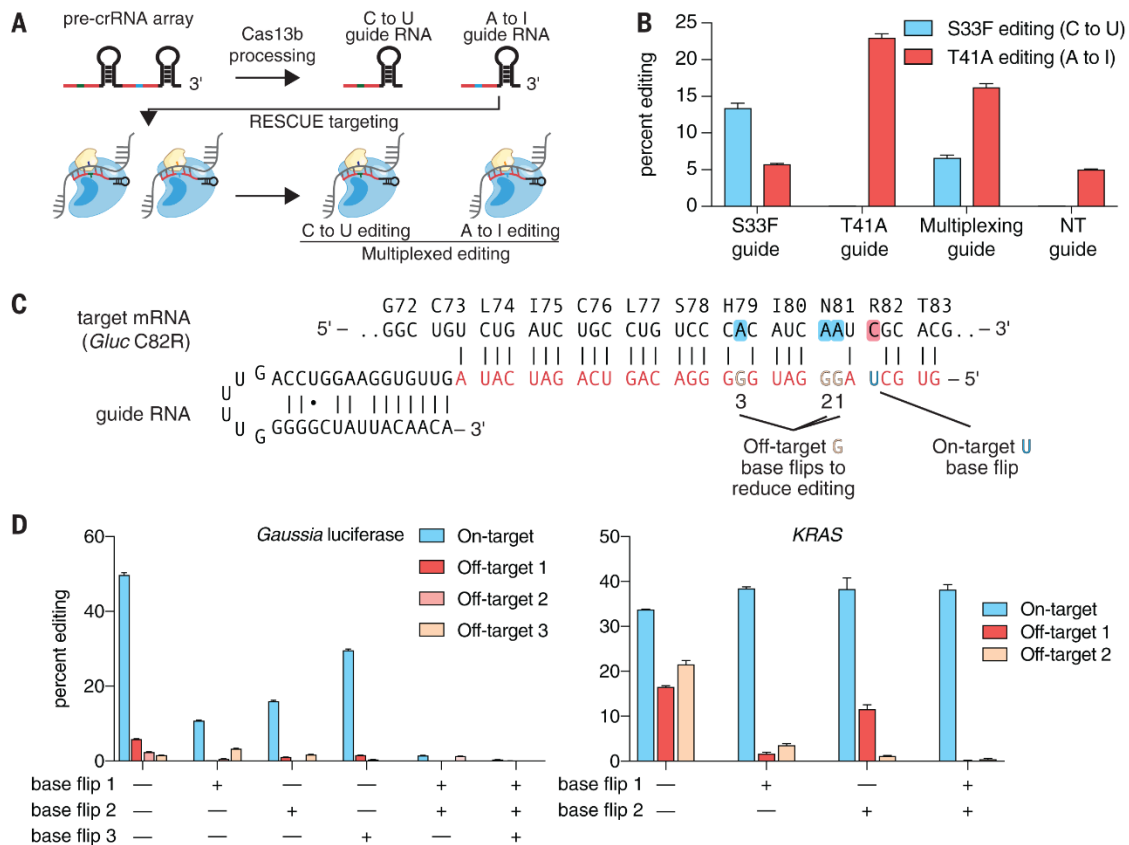


Fig. 3. RESCUE and REPAIR multiplexing and specificity enhancement via guide engineering. (A) Schematic of multiplexed C to U and A to I editing with pre-crRNA guide arrays. (B) Simultaneous C to U and A to I editing on *CTNNB1* transcripts. (C) Schematic of rational engineering with guanine base flips to prevent off-target activity at neighboring adenosine sites. (D) Percent editing at on-target C and off-target A sites for *Gaussia luciferase* (left) and *KRAS* (right) using rational introduction of disfavored base flips.

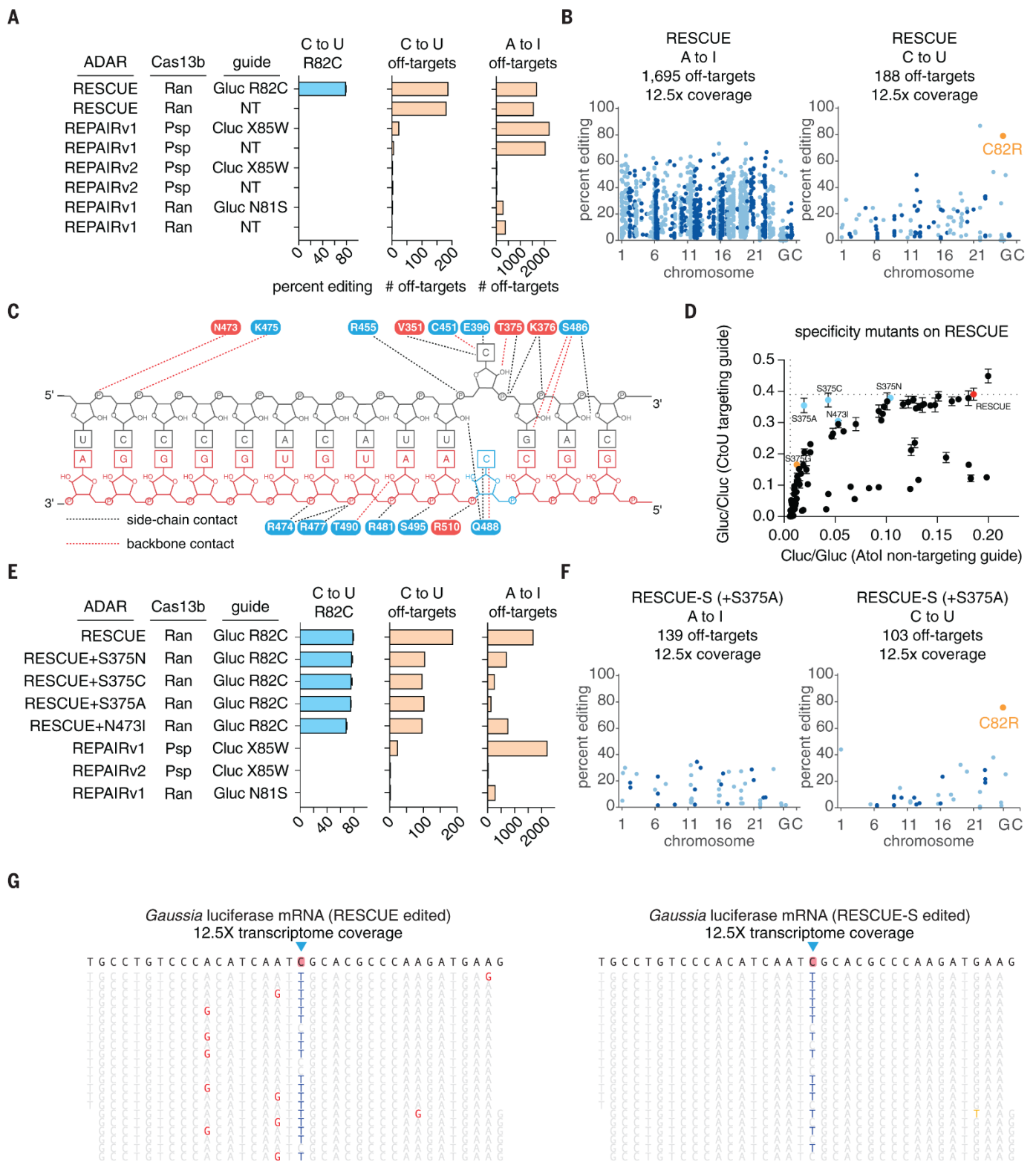


Fig. 4. Transcriptome-wide specificity of RESCUE. (A) On-target C to U editing and summary of C to U and A to I transcriptome-wide off-targets for RESCUE compared to REPAIR. (B) Manhattan plots of RESCUE A to I (left) and C to U (right) off-targets. The on-target C to U edit is highlighted in orange. (C) Schematic of ADAR2dd interactions with RNA. Residues mutated for improving specificity are highlighted in red. (D) Luciferase values for C to U activity with a targeting guide (y-axis) and A to I activity with a non-targeting guide (x-axis) shown for RESCUE and 95 RESCUE mutants. RESCUE is highlighted in red and mutants with better specificity in blue. The T375G mutant (REPAIRv2) is shown in orange. (E) On-target C to U editing and summary of C to U and A to I transcriptome-wide off targets of RESCUE, REPAIR, and top specificity mutants. (F) Manhattan plot of RESCUE-S (+S375A) A to I (left) and C to U (right) off-targets. The on-target C to U edit is highlighted in orange. (G) Representative RNA sequencing reads surrounding the on-target Gluc editing site (blue triangle) for RESCUE (left) and RESCUE-S (right). A to I edits are highlighted in red; C to U (T) edits are highlighted in blue; sequencing errors are highlighted in yellow.

A cytosine deaminase for programmable single-base RNA editing

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