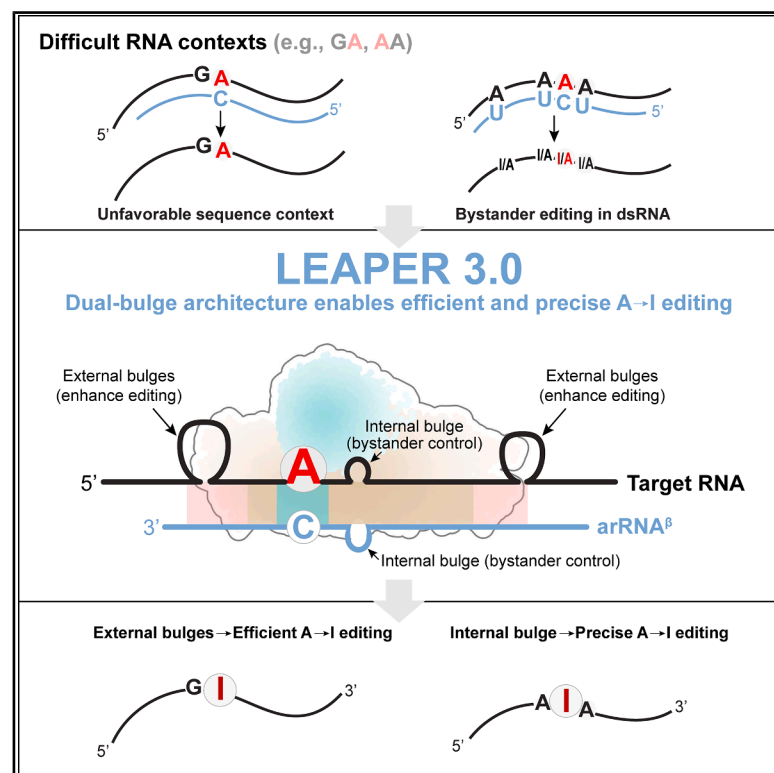


RNA structure programs endogenous ADAR for precise and efficient editing

Graphical abstract



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In brief

LEAPER 3.0, a structurally optimized dual-bulge ADAR-recruiting RNA design platform, achieves precise and efficient endogenous A-to-I RNA editing across diverse ADAR contexts.

Highlights

- Functional landscapes of ADAR1 or ADAR2 along arRNA-target RNA duplexes are defined
- RNA bulges within ADAR-binding regions modulate editing sensitivity and specificity
- Engineered bulges expand the editable RNA sequence space for endogenous ADAR editing
- LEAPER 3.0 enables precise and efficient RNA editing across diverse ADAR contexts

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Article

RNA structure programs endogenous ADAR for precise and efficient editing

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SUMMARY

Leveraging endogenous adenosine deaminase (ADAR) enzymes through engineered ADAR-recruiting RNAs (arRNAs) offers a safe, programmable strategy for RNA editing without exogenous enzyme delivery. Yet an incomplete understanding of ADAR's mechanistic basis has hindered the rational design of arRNAs with improved efficiency and precision. Here, we present LEAPER 3.0 (leveraging endogenous ADAR for programmable editing of RNA), a next-generation RNA-editing platform that integrates AlphaFold 3 structural predictions with systematic biochemical and cellular assays to define the molecular interface between ADAR1 or ADAR2 and double-stranded RNA. These insights enabled the rational optimization of arRNAs to expand the editable sequence range to previously refractory sites, suppress bystander editing within duplex regions, and achieve single-nucleotide discrimination among adjacent adenosines. This work elucidates the structural and mechanistic principles underlying arRNA-mediated editing and establishes a framework for the rational design of highly efficient and precise A-to-I RNA-editing tools.

INTRODUCTION

Unlike DNA editing, which results in permanent genomic alterations, programmable and site-specific RNA editing introduces reversible changes, offering unique advantages for therapeutic applications.¹ Recent advances in base editing have enabled targeted adenosine-to-inosine (A-to-I) and cytidine-to-uridine (C-to-U) conversions by using ectopically expressed engineered editors, including CRISPR-based systems^{2–4} and CRISPR-free ribonucleoprotein-based platforms.^{5–8} However, the overexpression of ectopic enzymes raises safety concerns, including widespread off-target effects^{9,10} and potential toxicity.¹¹

A promising approach to address these limitations is to leverage endogenous proteins for RNA editing. A-to-I editing, one of the most prevalent post-transcriptional modifications in eukaryotes, is mediated by endogenous adenosine deaminases (ADARs) acting on double-stranded RNA.^{12–14} By using chemi-

cally synthesized or plasmid-borne ADAR-recruiting RNAs (arRNAs), several technologies, developed by us and others, have successfully harnessed endogenous ADAR to enable site-specific RNA editing.^{11,15,16} Notably, a single dose of adeno-associated virus (AAV-delivered arRNA has achieved up to 50% editing efficiency in the livers of non-human primates (NHPs) for 13 weeks and around 20% editing in neurons of a murine Rett syndrome model, highlighting the therapeutic potential of this approach without relying on exogenous enzymes.^{17,18}

Despite these advances, key challenges continue to limit the clinical translation of endogenous RNA base editing. First, unlike the short guide RNAs used in CRISPR-Cas or small interfering RNA (siRNA) systems, arRNAs typically form extended duplexes (>100 bp) with their targets.¹⁵ While such long pairing is required for efficient editing, it often induces widespread bystander editing at adenosines throughout the duplex.^{19,20} The LEAPER system (leveraging endogenous ADAR for programmable editing of



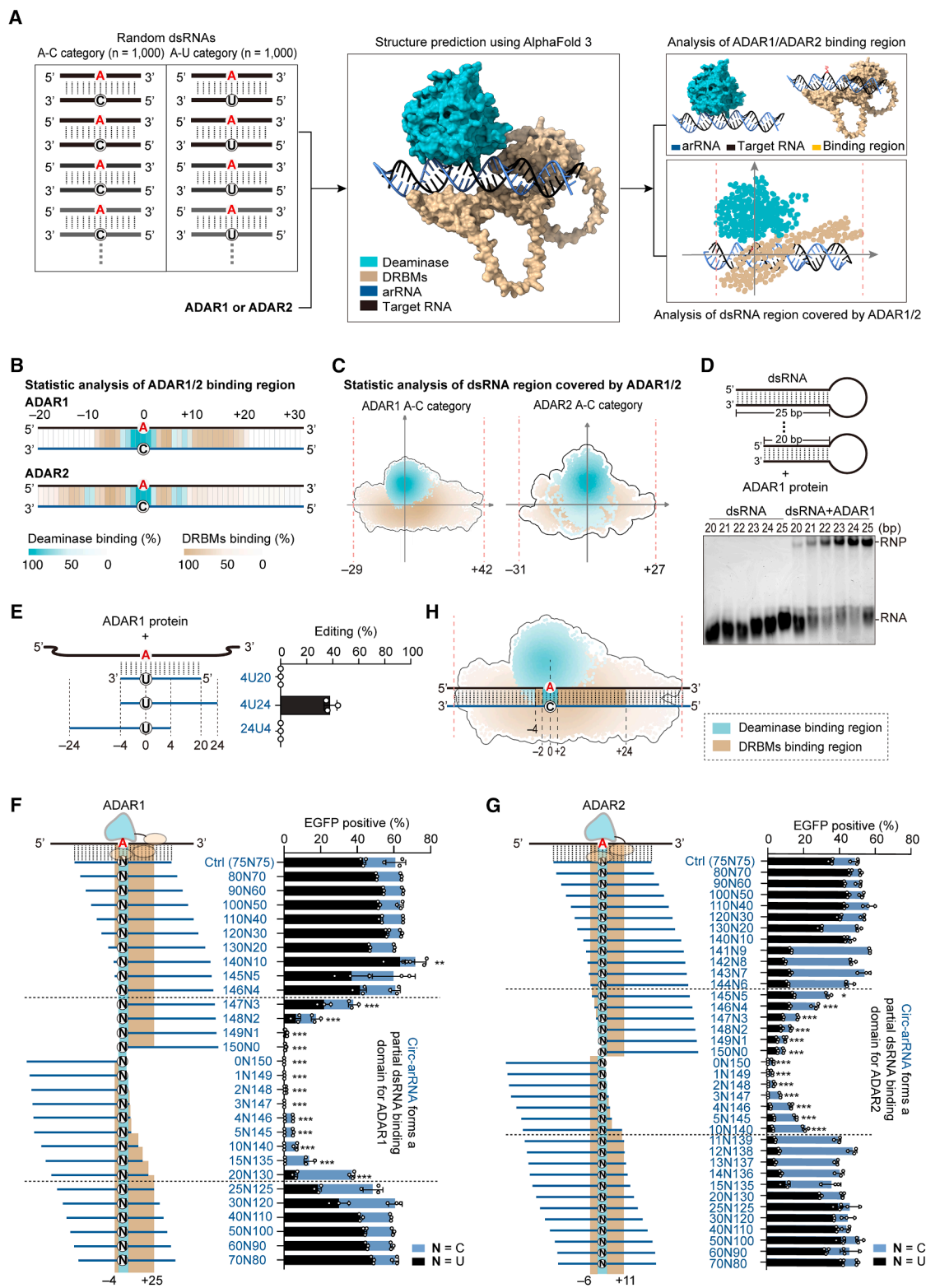


Figure 1. Integrated structural analysis of ADAR1 and ADAR2 using computational, *in vitro*, and cellular approaches

(A) Schematic diagram illustrating the workflow for structural prediction and analysis of the ADAR1-dsRNA and ADAR2-dsRNA complexes. See also Figures S1, S2, and S3.

(legend continued on next page)

RNA) mitigates these effects by introducing G mismatches or U deletions opposite bystander sites^{15,21}; however, these strategies become cumbersome when multiple adenosines are clustered within the same region. Second, ADAR's intrinsic sequence preference restricts efficient editing largely to 5'-UAN motifs,²⁰ leaving many disease-relevant sites, including those involving 5'-UGA or 5'-UAA nonsense mutations (accounting for ~60% of PTC [premature termination codon]-associated disorders), beyond reach.²² Third, insufficient editing precision can lead to adjacent bystander editing, where neighboring adenosines are inadvertently modified, a critical concern for applications requiring single-nucleotide specificity, such as the correction of the 5'-CAA codon in alpha-1 antitrypsin deficiency (AATD).^{23,24} Addressing these challenges requires a deeper mechanistic understanding of ADAR-double-stranded RNA (dsRNA) recognition and catalysis.

To this end, we employed AlphaFold 3 to model the structure of the ADAR1 or ADAR2 (ADAR1/2)-dsRNA complex, identifying key orientation and functional regions, including the deaminase catalytic interface, double-stranded RNA binding motifs (DRBMs) interface, and an ADAR-covered peripheral region that modulates substrate accessibility. Guided by these structural predictions, we performed a series of biochemical and cellular experiments to validate the molecular organization and dynamic behavior of ADAR1/2 on RNA duplexes. This integrative framework not only reveals the structural basis of ADAR1/2-dsRNA interactions but also enables rational optimization of arRNAs to achieve enhanced efficiency, reduced bystander activity, and single-nucleotide precision, even within refractory sequence contexts, thereby laying the foundation for next-generation endogenous RNA-editing therapeutics.

RESULTS

A critical binding interface formed by the deaminase and dsRNA-binding motifs governs A-to-I editing efficiency

A comprehensive structural understanding of the ADAR-dsRNA complex is essential for elucidating ADAR's biological function and for the rational design of effective arRNA. To this end, we employed AlphaFold 3²⁵ to model both the ADAR1-dsRNA and ADAR2-dsRNA complexes (Figure 1A). Unexpectedly, we observed notable variability in predicted structures across different runs, even when using the same dsRNA sequence (Figures S1 and S2). To circumvent this limitation, we adopted a statistical approach, analyzing thousands of AlphaFold

3-generated models to identify shared structural features that define ADAR1/2's interaction with dsRNA (Figures S1, S2, and S3). Rather than dissecting sequence-specific interactions, we hypothesized that this approach could uncover overarching principles governing ADAR1/2 recognition of dsRNA.

To delineate the key functional regions of ADAR activity within the long RNA duplex formed between arRNA and the target RNA, we combined structural modeling with statistical analysis of ADAR1/2-dsRNA complexes. Specifically, we delineated two regions of interest: the ADAR1/2-binding region, encompassing RNA bases that directly contact ADAR through structural mapping, and the ADAR1/2-covered region, determined by projecting the positions of ADAR1/2 α -carbon (C- α) atoms onto the RNA duplex to estimate the maximal spatial footprint engaged during catalysis (Figure 1A).

Although efficient editing typically requires long arRNA-target duplexes, our analysis revealed that the catalytically relevant ADAR1/2 interface occupies only a short segment within this extended structure (Figures 1B and 1C). Intriguingly, in both A-C and A-U editing contexts, the ADAR1-binding and covered regions were asymmetrically distributed, biased toward the 3' side of the edited site, in contrast to the more symmetrical binding patterns reported in prior ADAR2-dsRNA crystal structures²⁶ (Figures 1B and 1C; Figure S4A). However, unlike ADAR1, ADAR2 interacts with dsRNA through a shorter 3' interface and a more extended 5' contact region, suggesting a greater functional contribution from 5'-side RNA interactions (Figures 1B and 1C; Figures S4A and S4B).

To validate these *in silico* findings, we next examined the binding affinity of purified protein of ADAR1 to dsRNA *in vitro*. Binding assays revealed that a minimum duplex length of 24 base pairs was required for stable interaction (Figure 1D). However, when the target site was positioned near the 3' end of the duplex, editing efficiency dropped significantly, even with adequate duplex length (29 bp), suggesting that the adenosine had shifted into the DRBM-dominated region rather than the deaminase-binding region (Figure 1E), consistent with our structural predictions.

Interestingly, while 29 bp is sufficient *in vitro*, most arRNAs used in cellular systems are substantially longer to achieve efficient editing.^{15,27} We therefore hypothesized that within these extended duplexes, a core segment primarily mediates ADAR1/2 recruitment, while the flanking sequences mainly contribute to target recognition and can be configured with greater flexibility. To delineate this, we generated a panel of

(B) Heatmap summarizing predicted interaction sites of ADAR1 and ADAR2 on dsRNA substrates containing A-C target sites across all structural models. The corresponding heatmap for A-U target sites is shown in Figure S4A.

(C) Density scatterplot depicting the distribution of C α coordinates for the ADAR deaminase domain (blue) and DRBM domains (brown) relative to A-C target sites. See also Figure S4B.

(D) Electrophoretic mobility shift assay (EMSA) of dsRNAs of varying lengths following incubation with purified ADAR1 p110 protein.

(E) Sanger sequencing analysis of editing efficiencies using arRNAs paired with target RNAs of different lengths and orientations.

(F and G) Left: schematic illustrating the spatial arrangement of arRNA, target RNA, and ADAR1 (F) or ADAR2 (G). Blue shading indicates the region bound by the deaminase domain, and brown shading indicates the DRBM-binding region. Right: fluorescence-activated cell sorting (FACS) analysis of enhanced green fluorescent protein (EGFP)-positive cells, reflecting editing activity for the corresponding arRNA designs ($n = 3$). Data were analyzed by one-way ANOVA; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

(H) Proposed model of ADAR1 binding to its dsRNA substrate. The corresponding model for ADAR2 is shown in Figure S4C.

See also Figures S1, S2, S3, and S4.

circular arRNAs (LEAPER 2.0) with fixed duplex lengths but systematically shifted alignment positions relative to the target site in wild-type HEK293T cells (Figure 1F) and in HEK293T-ADAR1^{-/-} cells (Figure 1G). Using a dual-fluorescence reporter assay,¹⁵ we observed that strong editing occurred when the arRNA maintained a relatively shorter duplex region in both the ADAR1-dominant (wild-type HEK293T) and ADAR2-enriched (HEK293T-ADAR1^{-/-}) settings.

Although A-U contexts require a slightly longer 3' duplex than A-C contexts, the overall ADAR1 binding window remained consistent across both pairing backgrounds (Figure 1F). By contrast, the ADAR2-dependent window shifted substantially: in A-U contexts, efficient editing required an intact -10 to +20 bp duplex, whereas in A-C contexts this compressed to -6 to +11 bp (Figure 1G).

These results indicate that A-C mismatches and A-U base pairs represent fundamentally different experimental conditions for probing ADAR activity and for optimizing arRNA design. Given that A-C mismatches have become a standard arRNA enhancement strategy, we defined the core ADAR1/2 binding window based on the A-C-supported duplex range, providing a more generalizable framework for RNA-editing applications.

Together, our structural predictions and functional assays support a unified working model of ADAR1-dsRNA or ADAR2-dsRNA interaction (Figure 1H; Figure S4C). In this model, the DRBMs and deaminase domain align asymmetrically on the dsRNA, establishing a composite binding interface that spans from -4 to +24 (ADAR1) or -6 to +11 (ADAR2) around the editing site. This region is essential for stable ADAR1/2 binding and for achieving efficient A-to-I editing and thus represents a key target for optimizing arRNA design.

Enhanced sensitivity of A-U base-paired sites to RNA bulges within the ADAR1 DRBM-binding region

To further dissect the role of ADAR's binding regions in regulating RNA editing, we systematically examined how the RNA structure within the binding domains affects editing efficiency. Using a panel of engineered circular arRNAs (LEAPER 2.0) containing various RNA bulges at distinct positions and types (Figures 2A–2C), we evaluated editing outcomes across different RNA bulges. Disruptions introduced within the deaminase-binding region, either proximal to (Figures S5A and S5B) or directly overlapping the editing site (Figure S5C), markedly reduced editing efficiency at both A-U and A-C paired sites. These results underscore the critical importance of maintaining duplex integrity within the deaminase-binding region for efficient ADAR-mediated editing (Figure S5D).

We next investigated the functional impact of RNA bulges within the DRBM-binding region. Interestingly, in contrast to A-C mismatched target sites, A-U base-paired sites exhibited significantly heightened sensitivity to bulges positioned within the DRBM-associated region (e.g., at +3, +11, +16, and +21) (Figure 2D; Figure S6A). These structural disruptions led to a broad and sustained suppression of editing activity, which persisted until the bulge was moved beyond the DRBM-binding region (e.g., +26) (Figure 2D). Notably, even small bulges (1/0 or 0/1 bulges) introduced within this region substantially impaired

editing efficiency across a wide range of downstream positions on A-U arRNAs.

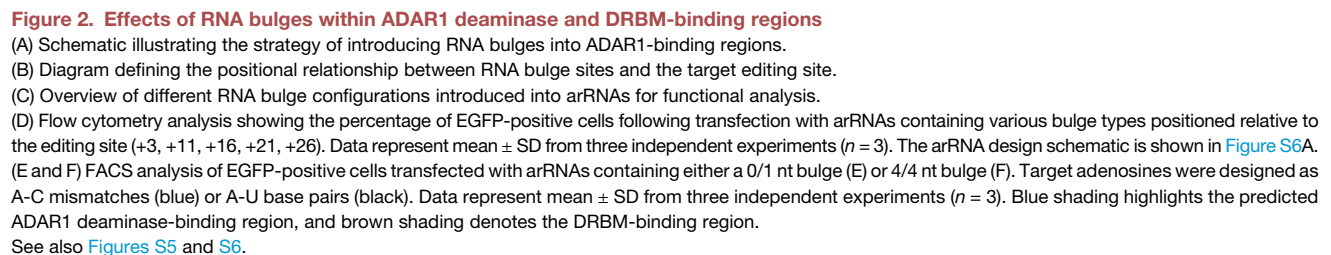
To more precisely map the effect of bulge size and position, we introduced 1/0 or 4/4 nucleotide bulges across a defined window (from -30 to +30 or -8 to +30, respectively) (Figures 2E and 2F). While bulges on the 5' side of the editing site resulted in similar editing patterns between A-U and A-C duplexes, bulges positioned on the 3' side only led to a pronounced drop on editing activity specifically for arRNAs with A-U base pairing. Remarkably, even a minimal 1/0 bulge within positions +1 to +25 caused a substantial loss of editing efficiency (Figure 2E), and this inhibitory effect became more pronounced with increasing bulge size (Figure 2F). These findings indicate that structural disruptions within the DRBM-binding region preferentially impair editing at A-U pairs, whereas A-C mismatches remain largely unaffected (Figure S6B).

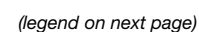
To confirm that these effects were intrinsic to the ADAR1-RNA interaction and not mediated by other cellular factors, we performed *in vitro* assays using purified ADAR1 p110 protein and *in vitro*-transcribed RNA substrates (Figure S6C). Consistent with *in vivo* results, A-U arRNAs exhibited a progressive recovery in editing activity only when the bulge was positioned beyond +25, supporting the idea that ADAR1's sensitivity to structural perturbations is influenced by base-pair context. Together, these results suggest that A-U base-paired editing sites are inherently more susceptible to disruptions within the DRBM-binding region, likely reflecting differential interaction dynamics between ADAR1 and A-U versus A-C duplexes.

A-C mismatches exhibit stronger deaminase engagement and greater tolerance to structural disruptions within the DRBM-binding region

To elucidate how local RNA distortions affect ADAR1-dsRNA interactions, we used AlphaFold 3 to simulate the structural consequences of inserting RNA bulges into defined positions of the duplex. Drawing from our previous large-scale structural predictions, we selected 106 representative dsRNA duplexes from each of the 1,000-member A-C and A-U groups, differing only at the nucleotide (C or U) opposite the editable adenosine. In both contexts, baseline structures consistently showed strong deaminase engagement with the target adenosine prior to bulge insertion. We then evaluated how these interactions were altered following bulge insertions at specific sites (Figure 3A).

Insertion of a 5/0 nucleotide bulge at position +2, located within the deaminase-binding region, completely abolished deaminase-adenosine contact in both A-C and A-U complexes, confirming that local duplex integrity at the editing site is indispensable for catalysis (Figure 3B, left). By contrast, when the bulge was positioned within the DRBM-binding region (e.g., a 5/0 bulge at +11), structural outcomes diverged sharply between A-C and A-U substrates (Figure 3B, right). Although AlphaFold 3 continued to generate stable ADAR1-dsRNA complex models for both categories, the predicted DRBM-binding regions shifted away from the bulged site toward adjacent intact duplex regions (Figure 3C). These rearrangements paralleled our *in vitro* findings showing that DRBM-region bulges markedly reduce ADAR1 binding affinity to dsRNA (Figures S7A and S7B).





Together, these findings suggest that RNA bulges primarily perturb DRBM-mediated contacts, while differential editing efficiencies between A-C and A-U substrates likely reflect distinct deaminase affinities for mismatched versus base-paired adenosines. Detailed structural analyses further supported this model: whereas both duplex types exhibited robust deaminase binding before bulge insertion, only A-C mismatches retained deaminase engagement afterward (Figure 3D; Video S1). Consistently, the deaminase-RNA contact interface was broader and more extensive in A-C mismatches than in A-U pairs (Figure 3E), corroborating earlier observations of an expanded deaminase-binding footprint at A-C sites (Figure 1B; Figure S4A).

Collectively, these findings demonstrate that although bulges within the DRBM-binding region disrupt overall ADAR1-dsRNA association, A-C mismatches maintain stable deaminase interaction and preserve editing activity, whereas A-U pairs are more susceptible to structural perturbation. This differential resilience provides a mechanistic explanation for the preferential editing of A-C substrates and offers a structural framework for engineering arRNA with single-nucleotide precision.

A bulge-guided strategy for precision RNA editing by targeting DRBM-binding regions

Based on the findings from Figure 2, we observed that introducing RNA bulges within either the deaminase or DRBM-binding regions significantly reduces editing efficiency at A-U base-paired bystander sites. When analyzing the inhibitory range using the RNA bulge as the reference point (Figures 2E and 2F; Figure S5C), we found that a single RNA bulge is sufficient to suppress editing at A-U bystander sites across a span of at least 24 base pairs within the arRNA-target duplex (Figure 4A). This suggests that a small number of strategically placed bulges can effectively block a broad set of undesired bystander edits.

Furthermore, the marked difference in bulge sensitivity between A-U and A-C sites within the DRBM-binding region offers a unique opportunity to achieve single-nucleotide precision editing by modifying only the arRNA design. We hypothesized that placing an RNA bulge outside the essential deaminase-binding window of the on-target site (−2 to +2) would minimally affect editing efficiency at an A-C mismatch site. By contrast, the same bulge would fall within the DRBM or even deaminase-binding regions of neighboring A-U bystander sites, thereby selectively reducing their editing efficiency (Figure 4B).

To evaluate this precision editing strategy, we tested it at the 5'-UAAAG target site using a wild-type HEK293T reporter system. In this setup, we sequentially designated each of the three consecutive adenosines as the intended editing site.

For example, when the first adenosine was targeted, only transcripts exhibiting a UIAA sequence in next-generation sequencing (NGS) reflected successful high-precision editing, whereas alternative outcomes such as UIIA, UIII, UAII, or UAIA were considered undesired adjacent bystander edits. Our results showed a high frequency of bystander editing across all three targeting scenarios when only an A-C mismatch was introduced in the arRNA. However, the insertion of a 4/4 nucleotide bulge within the DRBM-binding region significantly suppressed editing at neighboring A-U sites while maintaining the editing efficiency at the intended A-C site (Figures 4C–4E).

We next extended this strategy to a therapeutically relevant context—AATD, a genetic disorder caused by mutations in *SERPINA1*, most severely represented by the PiZZ genotype. This genotype encodes a Glu342Lys substitution (GAG to AAG), resulting in misfolded alpha-1 antitrypsin protein that accumulates in hepatocytes and leads to liver and lung pathologies.^{23,24} Thus, achieving precise A-to-I editing to convert the disease-causing 5'-CAAG codon to 5'-CIAG requires single-base resolution to avoid edits at adjacent bystander sites (Figure 4F).

To evaluate the bulge-based precision editing strategy in this setting, we engineered a series of circ-arRNAs with either 1/0 or 4/4 nucleotide bulges and transfected them into HEK293T cells expressing the mutant *SERPINA1* transcript. Consistent with prior observations, bulges introduced within the DRBM-binding region selectively suppressed bystander editing without compromising on-target correction (Figures 4G and 4H). Notably, large bulges (4/4 nt) positioned between +10 and +15 can efficiently eliminate neighboring edits, while small bulges (1/0 nt), placed closer to the target site, were also sufficient to modulate specificity with minimal arRNA sequence changes.

These results demonstrate that leveraging the distinct bulge sensitivities of DRBM-binding regions enables a powerful arRNA design strategy to achieve precise, single-nucleotide RNA editing with minimal off-target activity.

Enhancing editing at 5'-GAN and 5'-CAN motifs by introducing RNA bulges within the ADAR1/2-covered peripheral regions of the dsRNA complex

Previous analyses identified the critical ADAR1/2 binding interface on dsRNA as essential for catalysis (Figures 1F and 1H), based on direct protein-RNA interactions. However, our structural analysis (Figure 1C; Figure S4B) also revealed an adjacent duplex segment within ADAR's maximal catalytic range but lying outside this conserved binding interface. We refer to this segment as the "ADAR-covered peripheral region" (highlighted

Figure 3. Structural modeling reveals differential stability of ADAR1-dsRNA complexes containing DRBM-region bulges

- (A) Workflow for structural prediction of the ADAR1-dsRNA complex containing bulge insertions.
(B) Quantification of predicted ADAR1 deaminase engagement with the target adenosine after introducing bulges at positions +2 or +11. Results are presented as the proportion of structural models showing retained or disrupted interactions.
(C) Heatmap summarizing predicted DRBM-dsRNA interaction sites across models containing bulge-disrupted duplex structures.
(D) Representative structural models comparing A-C mismatch (left) and A-U base-pair (right) contexts. Top row: overall structure before and after bulge insertion. Bottom row: 90° horizontally rotated view.
(E) Close-up view of deaminase interactions with the editing site, highlighting differential engagement between A-C mismatch and A-U paired contexts. See also Figure S7.

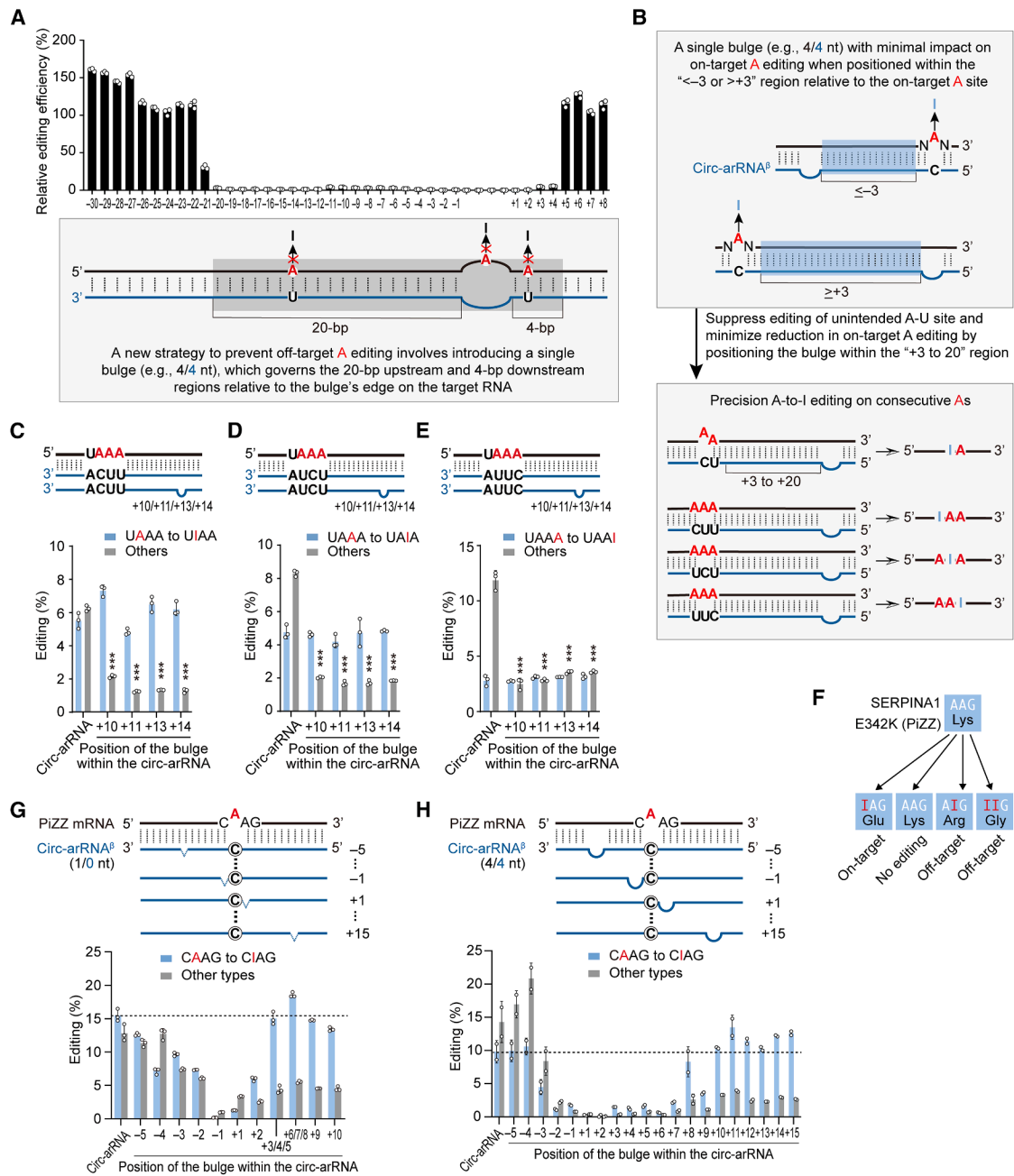


Figure 4. RNA bulges modulate the efficiency and specificity of ADAR1-mediated A-to-I editing

(A) Strategy for minimizing off-target editing through bulge insertion. Editing efficiency at A-U paired bystander sites is plotted relative to the RNA bulge position, with the bulge-defined center as reference. The shaded region indicates the effective suppression window for bystander editing.

(B) Diagram illustrating a precision editing strategy for sequences containing consecutive adenines. Bulges placed outside the core deaminase-binding window (-3 to $+3$) minimally affect on-target editing at A-C mismatches, whereas bulges positioned between $+3$ and $+20$ selectively suppress editing at adjacent A-U bystander sites.

(C–E) Quantification of precise editing outcomes converting UAAA to UAIA (C), UAIA (D), or UAAI (E). Target adenines (A-C mismatch) are shown in blue, and unintended bystander edits (A-U pairs) are shown in gray. Each group contains a 4/4 nt bulge positioned at $+10$, $+11$, $+13$, or $+14$. Data represent mean $\pm$ SD from three independent experiments ($n = 3$). Statistical significance was determined by one-way ANOVA; *** $p < 0.001$.

(F) Schematic of A-to-I editing targeting the AAG codon of the pathogenic *SERPINA1* E342K (PiZZ) variant, highlighting the requirement for high-precision editing. (G and H) Editing specificity profiles for the PiZZ mutation using arRNAs containing either a 1/0 nt bulge (G) or 4/4 nt bulge (H) at different positions. Data represent mean $\pm$ SD.

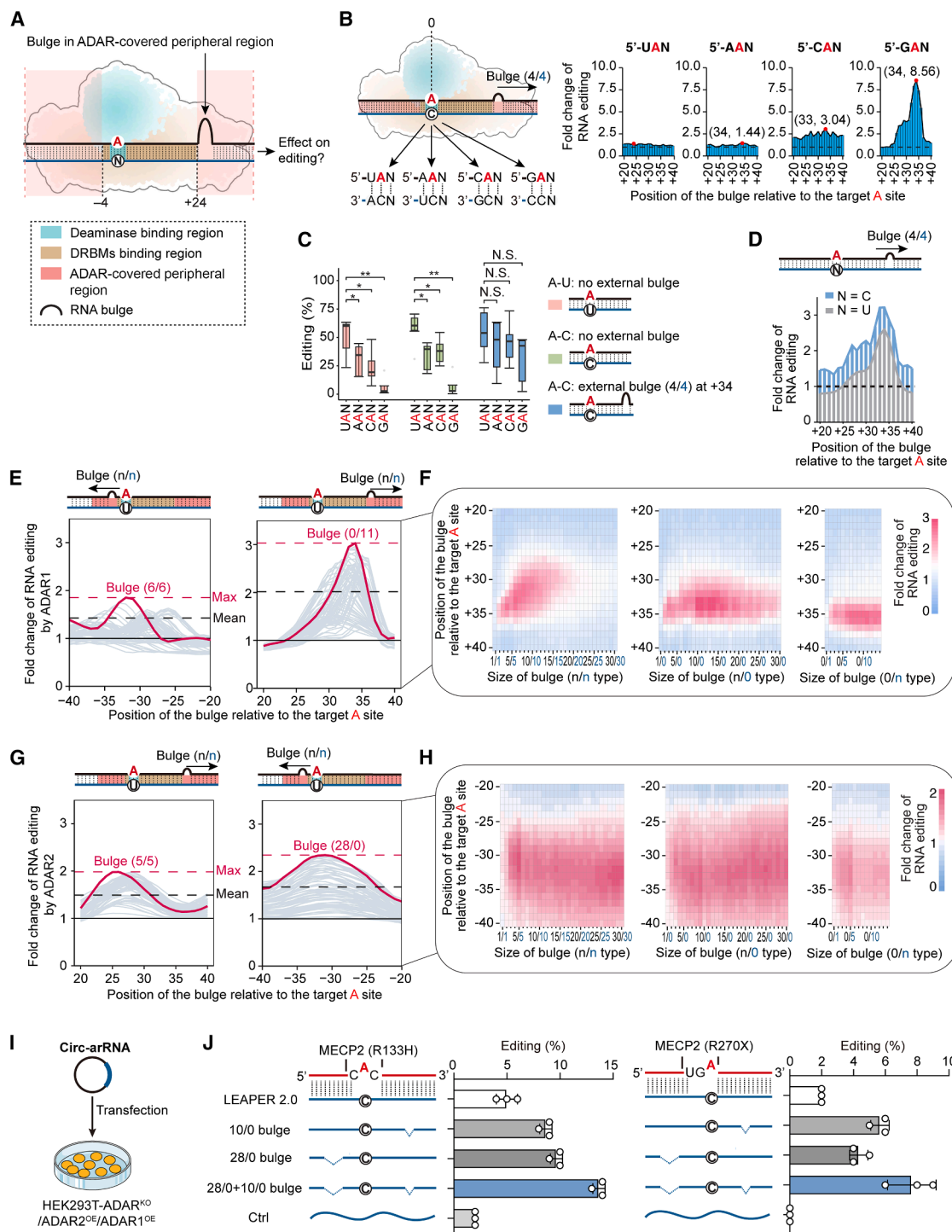


Figure 5. RNA bulges in ADAR1/2-covered peripheral regions enhance A-to-I editing efficiency

(A) Schematic of the ADAR1-dsRNA complex. The brown-shaded region indicates dsRNA segments directly contacting ADAR1, whereas the pink region represents the additional spatial coverage of the deaminase and DRBM domains.

(B) Editing fold-change analysis following insertion of a 4/4 nt bulge at the 3' boundary of the ADAR1-dsRNA interaction region. Editing outcomes are categorized by the 5' neighboring nucleotide of the target site (UAN, AAN, CAN, or GAN). Data were obtained from library screening. The screening workflow and site distribution are shown in Figures S8A and S8B.

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in pink in Figure 5A). Although both cellular and *in vitro* protein assays (Figure 1) indicated that complete removal of this region does not significantly impair basal ADAR activity, we hypothesized that it may nonetheless contribute to modulating ADAR engagement and editing efficiency.

To test this, we constructed a 151-nt dsRNA library and transfected it into HEK293T cells. This library contained evenly distributed adenosines, with select positions modified by inserting RNA bulges into the ADAR-covered peripheral region of the dsRNA (Figures S8A and S8B). By comparing editing efficiencies at each site with and without bulge insertion, we mapped the positional impact of RNA bulges on A-to-I editing (Figure S8A, data analysis). Strikingly, when editing sites were stratified by their 5' nearest-neighbor nucleotide, insertions within the ADAR-covered peripheral region markedly enhanced editing at 5'-CAN and 5'-GAN sites, which are typically resistant to endogenous ADAR editing (Figure 5B; Figure S8C).

Further analysis showed that, in the absence of bulges, ADAR1 exhibits its well-known preference hierarchy (5'-UAN > AAN > CAN > GAN) (Figure 5C; A-U: no external bulge). While introducing an A-C mismatch improves overall editing, it does not alter this intrinsic sequence preference (Figure 5C; A-C: no external bulge). Remarkably, when a bulge was placed in the 3' ADAR-covered peripheral region of the A-C duplex, editing at 5'-GAN or 5'-CAN sites dramatically increased, approaching levels typically observed at 5'-UAN sites (Figure 5C; A-C: external bulge (4/4) at +34). This effect provides a strategic avenue for enhancing editing at conventional refractory sites.

To evaluate the translational potential of this approach, we designed arRNAs incorporating bulges into the 5' ADAR-covered peripheral region and applied them to clinically relevant targets, including 5'-UGA- or 5'-UAA-containing PTCs found in *MECP2* (Rett syndrome), *KCNJ13* (rod-cone dystrophy), and *DMD* (Duchenne muscular dystrophy) transcripts.^{28–30} Compared with control circ-arRNAs, the bulge-containing arRNAs significantly enhanced editing at these traditional difficult-to-correct PTC sites (Figures S8D and S8E).

To evaluate the generalizability of this structural strategy, we next examined the effects of 4/4 nt bulges in both A-C and A-U mismatch libraries. Although A-C and A-U sites displayed distinct editing behaviors when bulges were placed within the DRBM-binding region, both exhibited similar enhancement trends when the bulge was introduced into the ADAR-covered

peripheral region (Figure 5D). Although previous structural prediction analyses did not identify direct ADAR contacts within these regions (Figures 1A and 1B), our results indicate that local structural perturbations in the ADAR-covered peripheral region can actively contribute to and enhance ADAR-mediated editing.

Further structural modeling revealed that different bulge configurations (e.g., 1/0, 0/1, 4/4, etc.) generate markedly distinct tertiary conformations within the dsRNA duplex (Figure S9). This prompted the hypothesis that the magnitude of enhancement conferred by bulges in the ADAR-covered peripheral region may depend on bulge type and geometry, suggesting that systematic screening could identify optimal configurations for maximal ADAR activation.

To comprehensively explore this, we constructed a panel of A-U target libraries encompassing a wide range of bulge configurations, 0/n bulges (0/1 to 0/14), n/n bulges (1/1 to 30/30), and n/0 bulges (1/0 to 30/0), and evaluated them in both ADAR1- and ADAR2-dominant cellular settings (Figures S8F and S8G). In ADAR1-driven contexts, bulges introduced into the 3' ADAR-covered peripheral region yielded higher overall and peak editing efficiencies than bulges placed on the 5' side (Figure 5E). While the overall enhancement trend was conserved, the amplitude and positional dependence varied across bulge types (Figure 5F; Figures S10, S11, S12A, and S12B). Specifically, 0/n and n/0 bulges achieved maximal activity at positions 34 and 35, respectively, whereas the optimal position of n/n bulges shifted progressively toward the 5' end as bulge size increased (Figure 5F). Regarding bulge size, editing efficiency rose with increasing bulge length until reaching a threshold, beyond which further expansion diminished the enhancement effect (Figure S12B). Among all tested designs, 10/0–13/0 bulges yielded the strongest enhancement. Importantly, 0/n bulges, while comparably effective, do not increase the overall arRNA length, making them particularly advantageous for compact, high-efficiency arRNA design.

Interestingly, unlike ADAR1, ADAR2 showed stronger editing enhancement when an appropriately sized RNA bulge was placed in the 5' ADAR-covered peripheral region (Figure 5G). In terms of bulge type, ADAR2 favored n/n and n/0 configurations over 0/n bulges (Figure S12C). Although the optimal position and magnitude of enhancement varied with bulge size (Figure 5H), ADAR2, distinct from ADAR1, exhibited greater editing enhancement when the 5' covered peripheral region

(C) Average editing rates across the four 5' sequence contexts for target sites that are A-U paired, A-C mismatched, or A-C mismatched with an external 4/4 nt bulge at position +34. Data were derived from high-throughput screening. Statistical significance was assessed by one-way ANOVA, where ** denotes $p < 0.01$ and * denotes $p < 0.5$.

(D) Comparative analysis of the enhancement effect of a 4/4 external bulge across A-U and A-C contexts.

(E and G) Fold increase in editing efficiency for each bulge configuration introduced from the 5' (left) and 3' (right) side of the ADAR1-covered peripheral regions (E) or the 3' (left) and 5' (right) side of ADAR2-covered peripheral regions (G). Red lines highlight the bulge configurations producing the highest fold change at each boundary.

(F and H) Heatmap showing fold changes in editing efficiency following the introduction of different bulge types (n/n, n/0, and 0/n) across positions +20 to +40 within the 3' ADAR1-covered peripheral region or –20 to –40 within the 5' ADAR2-covered peripheral region. Data were derived from library screening using A-U paired target sites.

(I) Experimental workflow for evaluating editing efficiency of arRNAs containing single or dual external bulges in cells co-expressing ADAR1 and ADAR2, established by transfecting expression plasmids into ADAR-knockout HEK293T cells.

(J) RNA-editing efficiencies of arRNAs carrying a single external bulge (10/0 at position +34 or 28/0 at –31) or dual external bulges (10/0 at +34 combined with 28/0 at –31).

See also Figures S8, S9, S10, S11, S12, and S13.

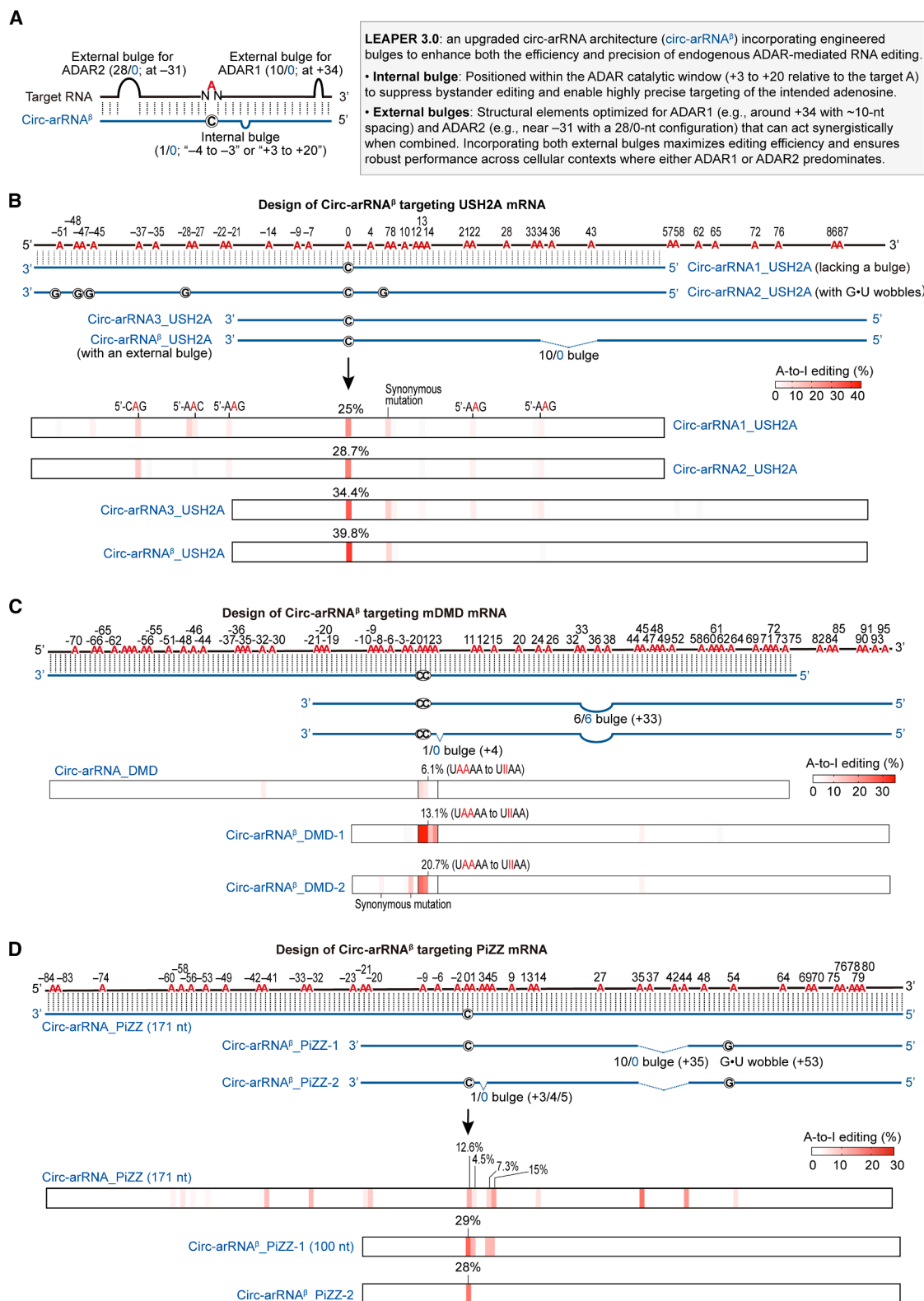


Figure 6. The arRNA[®] strategy enables precise and efficient A-to-I editing at disease-associated loci in HEK293T cells

(A) Schematic representation of the LEAPER 3.0 platform, highlighting the incorporation of internal and external RNA bulges to enhance editing precision and efficiency.

(legend continued on next page)

incorporated larger RNA structures, such as 30/30 or 28/0 bulges (Figure S12C). Compared with the control group, introducing a 28/0 bulge in the 5' covered peripheral region strongly enhanced arRNA-mediated editing in an ADAR2-dominant cellular environment (Figures S13A and S13B). Notably, the optimal bulge positions for ADAR1 and ADAR2 are symmetrically distributed along the duplex, suggesting that both structural requirements can be accommodated simultaneously in arRNA design. Consistent with this idea, arRNAs incorporating both the 28/0 and 10/0 bulge configurations exhibited improved editing efficiency at previously difficult-to-edit sites, regardless of whether cells expressed ADAR1, ADAR2, or mixed ADAR isoforms (Figures 5I and 5J; Figures S13C and S13D).

Rational design of circ-arRNA^β: A bulge-enhanced LEAPER 3.0 platform for efficient and precise RNA editing

Building on mechanistic insights into ADAR1/2 binding and bulge-induced modulation, we developed an optimized LEAPER 3.0 arRNA platform, termed arRNA^β (where “β” indicates the incorporation of bulge elements). This design integrates rationally placed internal bulges, a flexible dsRNA targeting region, and external bulges tailored to specific or mixed ADAR environments, collectively enabling improved on-target editing while minimizing bystander activity (Figure 6A).

To evaluate the therapeutic promise of this next-generation platform (LEAPER 3.0), we applied circ-arRNA^β to Usher syndrome, the most common genetic cause of combined deafness and blindness, affecting approximately 1 in 10,000 individuals globally.³¹ The USH2A 11864G>A mutation, a clinically significant nonsense variant associated with Usher syndrome type II (USH2), leads to progressive retinitis pigmentosa and moderate-to-severe hearing loss.³² As a 5'-UAG site, this locus is efficiently edited by the LEAPER 2.0 system. We therefore sought to determine whether the LEAPER 3.0 design, guided by mechanistic insights into ADAR function, could effectively suppress widespread bystander events while preserving high on-target activity at the USH2A 11864G>A site. As an initial design, we developed a 111-nt circular arRNA (Circ-arRNA1_USH2A), which achieved ~25.3% A-to-I editing at the USH2A c.11864G>A site in HEK293T (Figure 6B). However, despite its on-target efficiency, this design also triggered considerable bystander editing. Recent studies have shown that introducing 5'-G-U or 3'-G-U wobble base pairs into LEAPER arRNAs can suppress bystander editing at certain motifs, such as 5'-UAN and 5'-AAU triplets (where N = any base). However, this strategy is ineffective for suppressing editing at motifs like 5'-CAG, 5'-AAC, and 5'-AAG, which are prevalent in the USH2A transcript (Circ-arRNA2_USH2A).¹⁸

Guided by the ADAR1 binding map established in Figure 1, we redesigned the circ-arRNA by shifting the targeting region toward the 3' side of the editing site (Circ-arRNA3_USH2A), effectively distancing it from nearly half of the original bystander sites, resulting in improved editing efficiency. Finally, leveraging the arRNA^β design, we inserted a 10/0 bulge to selectively inhibit the remaining bystander site. Moreover, because this bulge was positioned within the ADAR-covered peripheral region of the dsRNA, it not only reduced residual off-target editing but also further enhanced on-target efficiency (Circ-arRNA^β_USH2A).

Similar to UGA stop codons, UAA codons are intrinsically resistant to ADAR-mediated editing. Unlike UGA, however, successful readthrough of a UAA stop codon requires simultaneous editing of two consecutive adenosines. Editing only the first adenosine converts UAA into UGA, generating a 5'-GAN motif that is even less preferred by ADAR and thus further diminishes overall readthrough efficiency. To evaluate whether LEAPER 3.0 could overcome this intrinsic limitation, we targeted a UAA stop codon in a mouse DMD sequence. Without any RNA bulge, conventional arRNA achieved only ~6% conversion in HEK293T cells (Figure 6C, Circ-arRNA_DMD). Because inserting 10/0 bulge at +35 unexpectedly generated a poly(T) sequence, reducing U6 promoter transcription efficiency (Table S2). Guided by library screening results (Figure 5F; Figure S12B), an alternative 6/6 bulge was chosen and positioned at +33. The results showed that arRNA^β (Figure 6C, Circ-arRNA^β_DMD-1) markedly boosted editing efficiency, demonstrating that structural tuning within the ADAR-covered peripheral region can bypass ADAR's native sequence constraints. As this is a 5'-UAAAA site, accurate restoration requires editing only the first two adenosines among four consecutive A bases. Although this level of precision exceeds typical PTC correction needs, we used it to test whether internal bulges could complement external ones for single-nucleotide precision. Analysis of individual NGS reads confirmed that LEAPER 3.0 achieved both higher efficiency and superior editing precision (Circ-arRNA^β_DMD-1: 13.1%; Circ-arRNA^β_DMD-2: 20.7%).

We further examined the PiZZ locus, requiring an exact amino acid correction from 5'-CAAG to 5'-CGAG, to test whether LEAPER 3.0 could enhance precision while minimizing adjacent bystander edits. Conventional designs produced extensive off-target editing within the duplex (Figure 6D, Circ-arRNA_PiZZ). Repositioning the arRNA target and introducing a 10/0 bulge at +34 (Figure 6D, Circ-arRNA^β_PiZZ-1) preserved on-target efficiency while substantially reducing bystander edits at positions +14, +35, and +44, a pattern consistent with the broad bystander-suppressing effect of bulge insertion (Figure 2A). Incorporating additional internal 1/0 bulges

(B) Stepwise optimization of arRNAs targeting the USH2A c.11864G>A mutation. The heatmap shows mean A-to-I editing efficiencies for each construct. The optimal design is circ-arRNA^β containing a 10/0 bulge at +34. The +7 A-to-G mutation is synonymous. Data represent mean values from three independent experiments ($n = 3$).

(C) Stepwise optimization of arRNAs targeting the DMD C3197T mutation. The heatmap indicates mean editing efficiencies for each design. Dual-target editing rates are indicated. The optimal design includes dual A-C mismatches, a 1/0 internal bulge at +4, and a 6/6 external bulge at +33. A-to-G mutations at -2 and -8 are synonymous.

(D) Optimization of arRNAs for correcting *SERPINA1* PiZZ mutation (E342K). The optimal design harbors a 1/0 internal bulge at +3/+4/+5, a 10/0 external bulge at +35, and a G-U wobble at +53. The heatmap displays A-to-I editing rates across the target region from triplicate experiments in HEK293T cells ($n = 3$).

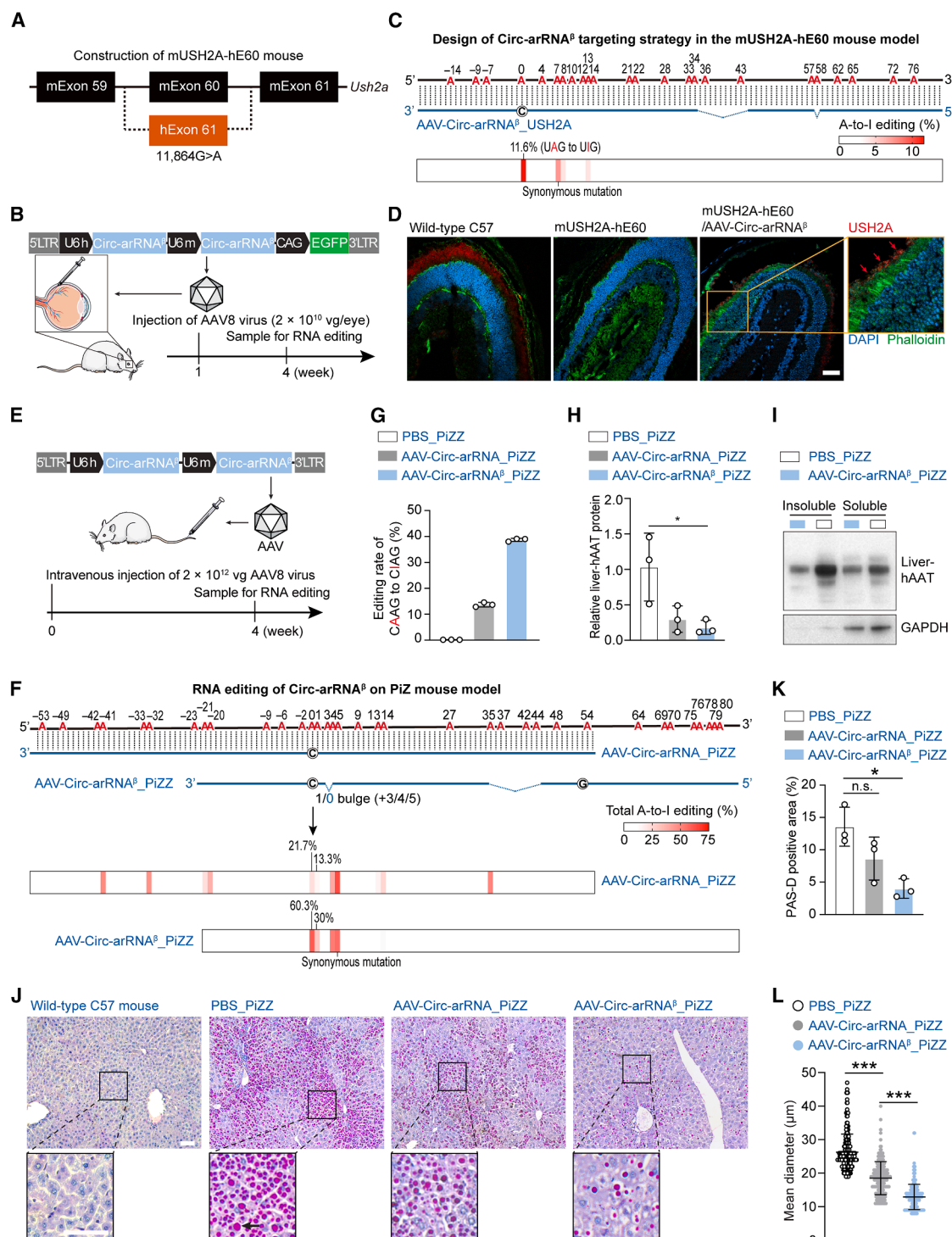


Figure 7. Therapeutic evaluation of LEAPER 3.0 in mouse models of Usher syndrome and AATD

(A) Generation of a humanized USH2A-hE61 mouse model carrying the clinically relevant USH2A 11864G>A mutation.

(B) Workflow for subretinal delivery of AAV8-packaged arRNA into Ush2A-hE60 mice.

(C) *In vivo* editing efficiency 4 weeks after injection. Heatmap and annotated values show mean A-to-I editing rates at the target and bystander sites covered by arRNA ($n = 3$). A-to-G mutation at position +7 is synonymous.

(D) Immunofluorescence images of retinal sections from wild-type mice, untreated mUSH2A-hE60 mice, and AAV-circ-arRNA[®]-treated mUSH2A-hE60 mice at 28 days post-treatment, showing partial restoration of usherin protein expression. Red arrows mark the positive staining signals of usherin protein. Scale bar, 50 μ m.

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at +3, +4, or +5 further minimized adjacent bystander events (Figure 6D, Circ-arRNA^β_PiZZ-2).

Collectively, these results demonstrate that the arRNA^β framework, through the coordinated use of internal and external bulges, enables high-efficiency, single-nucleotide-precise RNA editing at multiple therapeutically relevant loci, representing a versatile and generalizable strategy for transcript correction.

Circ-arRNA^β demonstrates therapeutic efficacy *in vivo* in Usher syndrome and AATD mouse models

To assess the *in vivo* therapeutic potential of circ-arRNA^β, we evaluated its activity in an mUSH2A-hE60 mouse model of Usher syndrome. To recapitulate the human USH2A c.11864G>A nonsense mutation, mouse exon 60 was replaced with the human exon 61 sequence harboring the corresponding PTC mutation (Figure 7A). An AAV8 vector co-expressing circ-arRNA and GFP was delivered by subretinal injection, enabling robust transduction and expression in retinal tissues (Figure 7B). GFP fluorescence confirmed efficient vector expression (Figure S14A), and retinal section imaging indicated that the retinal architecture remained intact post-injection, with no detectable structural abnormalities (Figure S14B). Because mUSH2A-hE60 mice did not exhibit a detectable difference in visual performance compared with wild-type controls at 15 months (Figure S14C), subsequent analyses focused on evaluating RNA-editing efficiency and protein expression following AAV treatment. RNA-editing analysis revealed strong editing in all mice treated with circ-arRNA^β, achieving an average efficiency of 11.6% (Figure 7C; Figure S14D). Although modest increases in bystander editing were observed at positions +8 and +13 relative to human HEK293T cells (Figure 6B), circ-arRNA^β preserved its overall editing pattern across species and effectively suppressed most off-target events (Figure 7C). Immunostaining with an anti-usherin antibody targeting the C terminus confirming partial restoration of Ush2a protein expression in the retinas of treated mice (Figure 7D).

We next evaluated circ-arRNA^β in a humanized mouse model of AATD carrying the SERPINA1 Glu342Lys (PiZZ) mutation.³³ Although these mice retain endogenous murine Serpina1 and therefore do not exhibit pulmonary disease, the mutant human SERPINA1 forms insoluble hepatic aggregates, leading to liver injury that recapitulates the human AATD hepatic phenotype. To validate the cross-species compatibility of circ-arRNA^β, we tested it in HEK293T-ADAR^{KO}/mADAR1^{OE} cells. Under the murine ADAR1 environment, bystander editing at positions +1 and +4 increased modestly compared with human cells. Nevertheless, circ-arRNA^β also achieved over a 2-fold improvement in precise RNA-editing efficiency and main-

tained suppression of most bystander events within the arRNA-covered region (Figure S14E).

Based on these findings, we packaged the optimized arRNA^β construct into an AAV vector for *in vivo* delivery (Figure 7E). Consistent with the cellular data, circ-arRNA^β achieved a marked increase in on-target editing efficiency compared with earlier-generation constructs (Figure 7F). Single-read NGS analysis revealed that approximately 40% of edited transcripts achieved precise amino acid correction (Figure 7G). Restoration of the correct sequence shifted SERPINA1 protein from an insoluble to a soluble form, markedly reducing hepatic aggregation. Western blot analysis confirmed lower total SERPINA1 protein levels and a pronounced reduction in the insoluble fraction in mice treated with AAV-circ-arRNA^β compared with control groups (Figures 7H and 7I; Figure S14F).

Histopathological evaluation using periodic acid-Schiff (PAS) staining with diastase treatment (PAS-D)³⁴ further revealed a significant decrease in hepatic protein aggregates following circ-arRNA^β treatment (Figure 7J). Quantitative analysis demonstrated that both the size and total area of SERPINA1 aggregates were substantially reduced in AAV-circ-arRNA^β-treated mice relative to PBS and AAV-circ-arRNA_PiZZ controls (Figures 7K and 7L).

DISCUSSION

Compared with RNA-editing technologies that rely on exogenous deaminases, strategies harnessing endogenous enzymes offer distinct advantages in both safety and delivery efficiency. For instance, repairing the USH2A mutation using the exogenous dPspCas13-E488Q editor (~4.15 kb) requires a total construct, comprising promoter, guide RNA, and poly(A) elements, that surpasses the AAV packaging limit (~4.7 kb), often necessitating dual-AAV delivery.^{2,35} This not only increases production cost but also introduces variability, as editing depends on the coordinated expression of both vectors, resulting in inconsistent outcomes across individuals. By contrast, the LEAPER system requires less than 500 bp in total for the promoter and circRNA sequence, easily fitting within AAV capacity. Its compact size allows multiplex arRNA delivery (Figure 7D) and provides flexibility for future inclusion of additional functional modules. Remarkably, despite the absence of any exogenous enzyme, arRNA achieved an 11.6% editing rate in the retina of Usher mouse model, surpassing Cas13-E488Q-based editors delivered via dual-AAV (2.04%) or single-AAV (0.32%) systems.³⁵

Despite these intrinsic benefits, endogenous A-to-I RNA editing has been limited by global bystander activity, a narrow

(E) Workflow for treating humanized AATD mice using two AAV8 vectors carrying arRNAs with or without bulges via intravenous injection.

(F) Accurate editing rates at the target and adjacent sites. Data represent mean ± SD (n = 3).

(G) Quantification of alpha-1 antitrypsin (hAAT) protein levels in treated and untreated AATD mice.

(H) Western blot analysis of soluble and insoluble liver hAAT protein using an anti-hAAT antibody. *p < 0.05 by one-way ANOVA.

(I) Heatmap showing *in vivo* A-to-I editing rates at target and bystander sites. Mean exact editing rates are indicated (n = 3).

(J) Periodic acid-Schiff-diastase (PAS-D) staining of mouse liver. Black arrows indicate PAS-D-positive aggregates. Scale bar, 80 μm.

(K) Quantification of PAS-D-positive area percentages (n = 3). Each dot represents an individual mouse. *p < 0.05 by one-way ANOVA.

(L) Quantification of the 70 largest globules per mouse based on PAS-D staining, analyzed using Fiji. Each dot represents a globule measurement. **p < 0.01 and ***p < 0.001, indicating statistical significance relative to PBS and circ-arRNA groups (one-way ANOVA).

See also Figure S14.

editable scope, and difficulty in controlling neighboring adenosine edits. For engineered editors such as REPAIR (RNA editing for programmable A-to-I replacement) or RESCUE (RNA editing for specific C-to-U exchange), these issues can be mitigated through protein engineering.^{2,36} By contrast, for endogenous A-to-I RNA editing, such constraints have been considered inherent to the properties of ADAR and arRNA. Due to ADAR's flexible, multi-domain organization, the lack of full-length ADAR1-dsRNA complex structural information has further impeded rational design of arRNA.^{26,37–40} Whether endogenous RNA editing could overcome these fundamental barriers remained unclear.

Through integrative analysis combining AlphaFold3-guided modeling, cellular and biochemical assays, library screening, and *in vivo* validation, we delineated key parameters governing arRNA-ADAR1/2 interactions. We identified ADAR1/2-binding and -covered regions that correlate strongly with editing efficiency, extending beyond previously annotated ADAR1-binding segments.³⁹ Although this region remains incompletely resolved at atomic detail, it provides valuable mechanistic insight for rational arRNA optimization.

Bystander suppression can be achieved through rational selection of the arRNA binding region (Figure 1F) and by exploiting the global inhibitory effect of bulges on A-U sites (Figure 4A). Because A-C and A-U contexts differ in bulge sensitivity, internal bulges introduced within the ADAR-binding region allow single-nucleotide control over adjacent edits (Figure 4B). Moreover, inserting bulges within the ADAR-covered peripheral region can reprogram intrinsic sequence preferences of ADAR, expanding the accessible motif space for endogenous editing (Figure 5).

Previous studies of natural ADAR substrates, such as the GluA2 Q/R site, showed that distal loop elements at the 3' end of the target RNA can enhance ADAR2 editing.⁴¹ In addition, previous studies have shown that introducing mismatches at fixed intervals downstream of the target site can enhance ADAR activation, with optimal spacing of ~35 nt for ADAR1 and ~26 nt for ADAR2.^{42,43} While canonical ADAR motif preferences (e.g., 5'-UAN > AAN > CAN > GAN) were originally derived from perfectly paired dsRNA substrates,^{20,44} our results indicate that local RNA geometry critically shapes ADAR activity, enabling enhanced editing even at less favorable motifs such as 5'-CAN and 5'-GAN. Moreover, the extent of editing enhancement and the optimal placement of a bulge depend not only on its type and size but are also dictated by the specific ADAR isoform involved. Notably, ADAR2 preferentially responds to bulges positioned on the 5' side, whereas ADAR1 achieves superior enhancement with bulges on the 3' side (Figures 5E and 5G). These isoform-specific preferences allow the LEAPER 3.0 platform to seamlessly integrate both design strategies, enabling effective optimization across single-isoform or mixed ADAR expression contexts.

The LEAPER 3.0 framework integrates these principles into a unified, modular arRNA design strategy (Figure 6A). This framework enables efficient target-site enhancement and precise bystander suppression without requiring complex bulge patterns or fragmented arRNA.^{45,46} Unlike black-box deep-learning-based arRNA designs, LEAPER 3.0 is biologically interpretable, transparent, and readily adaptable.^{47,48} Using this approach,

we designed arRNA^β constructs for Usher, mDMD, and PiZZ models, covering diverse sequence contexts. Across all cases, arRNA^β exhibited robust and well-balanced editing both *in vitro* and *in vivo*.

Collectively, our results reveal that the ADAR1/2 editing landscape is far more programmable than previously appreciated. Through rational arRNA design, editing efficiency, precision, and target scope can all be systematically optimized, establishing a conceptual and technical foundation for next-generation endogenous A-to-I RNA editing.

Limitations of the study

All *in vivo* experiments in this study employed AAV vectors, which, while efficient for proof-of-concept studies, pose cost challenges for clinical translation. Exploration of alternative delivery systems may enhance therapeutic applicability of the arRNA^β framework. Furthermore, while our study shows that the functional effects of RNA bulges on ADAR activity are strongly isoform-dependent and can even vary subtly among homologous ADARs from different species, most analyses here were focused on human ADAR1, which is highly expressed in HEK293T cells, as well as in eye and liver tissues (Figure S15). Sequence differences between ADAR1, ADAR2, and ADAR orthologs from other species likely underlie the distinct responses to RNA bulges observed across isoforms. By concentrating on RNA substrates, we established an experimental framework that serves as a broadly applicable platform for investigating ADAR mechanisms and optimizing arRNA designs. Future studies incorporating ADAR sequence variation may offer complementary insights into RNA substrate recognition and shed light on disease mechanisms associated with ADAR mutations.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents and resources should be directed to, and will be fulfilled by, the lead contact, Wensheng Wei (wswei@pku.edu.cn).

Materials availability

Reagents generated in this study are available upon request from the lead contact.

Data and code availability

Data in this study will be shared by the lead contact upon request. Sequence annotations and construct boundaries used in this study are as follows: the ADAR1 p110 (UniProt: P55265-5) deaminase domain spans residues 531–931, and the DRBMs are defined as DRBM1: 208–276, DRBM2: 319–387, and DRBM3: 431–499. The ADAR2 (UniProt: P78563) deaminase domain spans residues 299–701, and the DRBMs are defined as DRBM1: 78–144 and DRBM2: 231–298. For the constructs used here, non-core N-terminal regions were removed as follows: ADAR1 Δ1–207 and ADAR2 Δ1–81. All data used to generate line graphs and bar charts, as well as the original gel images, are compiled in Table S4. The raw sequencing data generated from the dsRNA library screening experiments have been uploaded to the National Genomics Data Center (<https://ngdc.cncb.ac.cn>).

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AUTHOR CONTRIBUTIONS

W.W. supervised the project. D.S., G.L., W.Z., and W.W. conceived the study. D.S., G.L., W.Z., H.T., and Y.L. designed the biological experiments. D.S., G.L., W.Z., and L.W. performed arRNA experiments in HEK293T cells with assistance from Y.Y., H.T., and Zongyi Yi. Zexuan Yi, Z.L., W.Z., G.L., and D.S. optimized arRNA designs for multiple disease targets. D.S., J.X., Y.L., Y.Z., and Y.S. conducted dsRNA library screening and performed data analysis. Y.S. and D.S. carried out protein purification and *in vitro* assays. P.Y. and Zexuan Yi constructed the mUSH2A-hE60 mouse model. H.T. and Zongyi Yi provided circ-RNA plasmid vectors for the construction of circ-arRNAs and conducted preliminary experiments on the mUSH2A-hE60 mouse model. D.S., G.L., J.X., S.Q., Zexuan Yi, H.L., G.T., P.Y., and W.X. designed and conducted the animal experiments. D.S. and J.R. conducted structural analyses with support from X.J. D.S., G.L., W.Z., and W.W. wrote the manuscript with input from Y.L., L.W., J.R., and Y.S.

DECLARATION OF INTERESTS

A patent application has been filed by Changping Laboratory (with W.W., D.S., and G.L. as inventors). W.W. serves as a scientific advisor and founder of EdiGene and Theroma. P.Y., Zexuan Yi, H.L., G.T., Y.Z., and Z.L. are employees of Leaper Bio Inc.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-alpha antitrypsin	Abcam	Cat#ab207303; RRID: AB_3665874
anti-β-actin	Abcam	Cat#ab6276; RRID: AB_2223210
anti-ADAR1	Cell Signaling Technology	Cat#81284; RRID: AB_3073708
anti-GAPDH	EasyBio	Cat#BE0024; RRID: AB_3099674
anti-USH2A	Leaper Bio Inc	N/A
anti-rabbit IgG (H+L)	Thermo Fisher Scientific	Cat#A11011; RRID: AB_143157
Bacterial and virus strains		
BL21 Competent E. coli	NEB	Cat#C2530H
E.coli HST08 Premium Electro-Cells	TAKARA	Cat#9028
Biological samples		
Mouse retinal tissue	This paper	N/A
Mouse liver tissue	This paper	N/A
Chemicals, peptides, and recombinant proteins		
DNase I	NEB	Cat#M0303L
NA-Red dye	Beyotime	Cat#D0128
Dulbecco's modified Eagle's medium	Gibco	Cat#C11965500BT
Penicillin- Streptomycin	Solarbio Science & Technology Co., Ltd.	Cat#P1400
Fetal Bovine Serum	Biological Industries	Cat#04-010-1A
Fetal Bovine Serum	VivaCell BIOSCIENCES	Cat#C04001-500
Protease Inhibitor Cocktail	Sangon Biotech	Cat#C500071
SIM-SF	Sino Biological	Cat#MSF1
SIM-HF media	Sino Biological	Cat#MHF1
X-tremeGENE™ HP DNA Transfection Reagent	Roche	Cat#6366236001
PrimeSTAR GXL Premix	TAKARA	Cat#R051
Phusion™ High-Fidelity PCR Master Mix	NEB	Cat#M0531L
PEI	Proteintech	Cat#B600070
DAPI Fluoromount-G	SouthernBiotech	Cat#0100-20
CoraLite 488-Phalloidin	Proteintech	Cat#PF00001
Critical commercial assays		
HiScribe™ T7 High Yield RNA Synthesis Kit	NEB	Cat#E2040S
RNA Clean & Concentrator Kit	Zymo Research	Cat#R1018
ZR small-RNA™ PAGE Recovery Kit	Zymo Research	Cat#R1070
AMV First Strand cDNA Synthesis Kit	Sangon Biotech	Cat#B532445
Quick-RNATM MiniPrep	Zymo Research	Cat#R1055
HiScript® III 1st Strand cDNA Synthesis Kit	Vazyme	Cat#R312
Periodic Acid Schiff Diastase Stain Kit	Abcam	Cat#AB287866
Plasmid Plus Midi Kit (100)	QIAGEN	Cat#12945
DNA Clean & Concentrator-25	Zymo Research	Cat#D4034
DNA Clean & Concentrator-5	Zymo Research	Cat#D4014
Golden Gate Assembly Kit	NEB	Cat#E1602L
Deposited data		
Raw RNA-seq files	https://ngdc.cncb.ac.cn/	PRJCA059931

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
HEK293T	C. Zhang (Peking University)	N/A
High Five cells	J. Xiao (Peking University)	N/A
Sf21 cells	J. Xiao (Peking University)	N/A
Experimental models: Organisms/strains		
B6.Cg-Tg(SERPINA1*E342K)Z11.03Slcw/ChmuJ	The Jackson Laboratory	Strain #: 035411
C57BL/6	Charles River Laboratories (Beijing, China)	Strain #: 219
mUSH2A-hE60	Edigene Inc. (Beijing, China)	N/A
Oligonucleotides		
dsRNA sequence for AlphaFold 3, see Table S1	This paper	N/A
arRNA sequence, see Table S2	This paper	N/A
Library sequence, see Table S3	This paper	N/A
Recombinant DNA		
pR8.74	Addgene	Cat#22036; RRID: Addgene_22036
VSVg	Addgene	Cat#158233; RRID: Addgene_158233
pLenti-sgRNA-lib 2.0 backbone	Addgene	Cat#89638; RRID: Addgene_89638
Software and algorithms		
FlowJo software	BD Biosciences	https://www.flowjo.com/ ; RRID: SCR_008520
GraphPad Prism	GraphPad Software	https://www.graphpad.com/ ; RRID: SCR_002798
ZEISS ZEN Microscopy Software (Version 3.5.10)	Carl Zeiss Microscopy GmbH	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html
UCSF ChimeraX	RBVI, UCSF	https://www.rbvi.ucsf.edu/chimerax/ ; RRID: SCR_002959
AlphaFold (v3)	DeepMind / Isomorphic Labs	https://alphafoldserver.com/ ; RRID: SCR_025454
Other		
Flow cytometry: LSRFortessa	BD Biosciences	Model: LSRFortessa
Whole-genome sequencing: HiSeq X Ten	Illumina	Platform: HiSeq X Ten
Zeiss LSM 980 laser scanning confocal microscope	Carl Zeiss Microscopy GmbH	Cat#LSM 980
Gene Pulser Xcell Microbial System	Bio-Rad	Cat#1652662
Gel imaging: ChemiDoc Imaging System	Bio-Rad	Model: ChemiDoc Imaging System

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**Cell lines**

HEK293T cells (C. Zhang, Peking University) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). HEK293T and HEK293T-*ADAR1*^{-/-} cell lines were incubated at 37°C in a humidified atmosphere with 5% CO₂. Sf21 and High Five insect cells (J. Xiao, Peking University) were cultured at 27°C in SIM-SF medium (Sino Biological, MSF1) and SIM-HF medium (Sino Biological, MHF1), respectively. All cultures were supplemented with 0.05% Penicillin-Streptomycin (Solarbio Science & Technology Co., Ltd., #P1400).

Mice

The mUSH2A-hE60 mouse model was designed by Edigene Inc. (Beijing, China). B6.Cg-Tg(SERPINA1*E342K)Z11.03Slcw/ChmuJ mice (stock No. 035411) were purchased from The Jackson Laboratory as humanized AATD model mice. C57BL/6 mice (stock No. 219) were purchased from Charles River Laboratories (Beijing, China) as wild type control mice. All mice were bred under specific pathogen-free (SPF) conditions at Vitalstar Biotechnology Co., Ltd. (Beijing, China) with a 12-hour light/12-hour dark cycle, controlled

temperature ($22 \pm 1^\circ\text{C}$), and $50 \pm 5\%$ humidity. Age- and sex-matched mice were randomly allocated to experimental groups for subsequent experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at JOINN Laboratories (Suzhou) Co., Ltd (approval No. S-ACU25-0858) and Vitalstar Biotechnology Co., Ltd. (Beijing, China; approval No. VST-PREPA-SOP-19-01). The number of animals used in this study was designed according to the appropriate guidelines.

METHOD DETAILS

DNA template generation

Our reporters used dual-fluorescence with open secondary structure sequences between mCherry and EGFP on a pLenti-CMV-MCS-PURO backbone. mCherry and EGFP formed a fusion protein, with a A-rich sequence carrying a stop codon between them. A P2A sequence was inserted before EGFP. Disease model reporters replaced the A-rich sequences with pathogenic mutations in human disease-related genes or cDNAs.

The circ-arRNA-expressing vector was designed using the pLenti-sgRNA-lib 2.0 backbone (Addgene, #89638). The vector's replaceable region is flanked by BsmBI enzyme sites. Newly designed arRNAs were synthesized through primer extension using PrimeSTAR GXL Premix (TAKARA, #R051) and inserted into the vector between the two BsmBI sites via a Golden Gate reaction (NEB, #E1602L). Plasmids were amplified in BL21 Competent *E. coli* (NEB, #C2530H) and purified using Plasmid Plus Midi Kit (QIAGEN, #12945).

Cell line construction

Reporter-expressing cells were created by lentivirus infection, with virus generated by co-transfecting pLenti-CMV-reporter-PURO with pR8.74 (Addgene, #22036) and pVSVG (Addgene, #158233) into HEK293T cells. The virus is collected after 74 hours in the supernatant of the culture dishes. Post-infection, mCherry-positive HEK293T cells with minimal EGFP leakage were sorted by flow cytometry and cultured in single clones. HEK293T ADAR1^{-/-} cell line were generated in a previous study in our laboratory. The knockout genotype was confirmed by sequencing. ADAR1 and ADAR2 proteins were confirmed to be absent by western blot.¹⁵

Cell culture and transfection

HEK293T cells (gifted by C. Zhang, Peking University) was cultured in DMEM (Gibco, #11965092) with 10% fetal bovine serum (FBS) (VivaCell BIOSCIENCES, #C04001-500) and 1% penicillin-streptomycin at 37°C with 5% CO_2 . Cells were plated in 24-well plates (2×10^5 cells per well) with serum-free medium 12 hours before transfection with 1 μg arRNA plasmids. The ratio of exogenous reporter and arRNA plasmids is 1:10 (100 ng vs. 1 μg). The ratio of exogenous reporter, arRNA and overexpressed ADAR plasmids is 1:10:10 (50 ng vs. 500 ng vs. 500 ng). PEI (Proteintech, #B600070) was used as a transfection reagent for the experiments to conduct fluorescence detection. And X-tremeGENE HP DNA Transfection Reagent (Roche, #06366546001) for the experiments to conduct next generation sequencing (NGS). The cells were harvested for further analysis 48 hours post-transfection.

Expression and purification of ADAR p110

ADAR1 p110 was expressed in High Five cells and subsequently purified by affinity chromatography. The DNA encoding ADAR1 p110 was cloned into the pFastBac vector with an N-terminal 8 $\times$ His tag. Bacmids were generated using generated Bac-to-Bac system (Invitrogen). Sf21 insect cells were used for recombinant baculoviruses generation and amplification, with both Sf21 and High Five cells cultured at 27°C in SIM-SF (Sino Biological, #MSF1) and SIM-HF media (Sino Biological, MHF1), respectively, supplemented with 0.05% Penicillin-Streptomycin (Solarbio® Life Science, #P1400). For protein production, High Five cells at a density of 2.0×10^6 cells/mL were infected with 5 mL of recombinant baculoviruses per liter. After 60 hours, the cells were harvested by centrifugation at 2,500 rpm for 10 min at 4°C . The cell pellet was resuspended in 100 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM β -mercaptoethanol, and 1 mM phenylmethanesulfonylfluoride) and disrupted using an ultrasonic breaker (SCIENTZ) for 10 min. The lysate was centrifuged at 16,000 rpm for 1 hour at 4°C , and the supernatant was collected. ADAR1 p110 was isolated using Ni-NTA affinity purification and eluted in lysis buffer supplemented with 300 mM imidazole, further purified using a Superdex 200 Increase column (Cytiva), and stored in a buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2 M L-Arginine, and 0.5 mM TCEP.

In vitro transcription and RNA purification

In vitro transcription (IVT) templates were prepared by PCR with T7 promoter-containing primers. RNA was synthesized using the HiScribe™ T7 High Yield RNA Synthesis Kit (NEB, #E2040S) per the manufacturer's protocol. DNA templates were degraded with DNase I (NEB, #M0303L), and IVT products were purified and concentrated with the RNA Clean & Concentrator Kit (Zymo Research, #R1018), followed by a final purification with the ZR small-RNA™ PAGE Recovery Kit (Zymo Research, #R1070) after 10% Urea-PAGE.

In vitro deamination assay

In vitro deamination assays involved target RNAs (199 nt), arRNAs (55 nt), and ADAR1 p110. Purified arRNAs and target RNAs were mixed at a 3:1 molar ratio, denatured at 90°C for 5 min, and annealed by gradual cooling to room temperature. *In vitro* deamidation

reactions were carried out by incubating 100 nM ADAR1 p110 with 50 nM annealed RNA in a buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT) at a total volume of 20 μ L. Controls excluded ADAR1 p110. Reactions proceeded at 37°C for 8 min, were stopped by heating at 85°C for 5 min, and the resulting complementary DNA (cDNA) was synthesized via reverse transcription with the AMV First Strand cDNA Synthesis Kit (Sangon Biotech, #B532445). PCR amplification of the cDNA was followed by Sanger sequencing to analyze the RNA editing.

Gel mobility shift assay

Electrophoresis mobility shift assays (EMSAs) were used to estimate RNA binding by ADAR1 p110. Purified RNA stems were heated to 90°C for 5 min, cooled to room temperature, and incubated with ADAR1 p110. RNA-protein complexes (RNPs) were formed with 200 nM RNA and 200 nM ADAR1 were incubated in a buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 5% glycerol). Binding reactions were conducted at 37°C for 10 min and run on a native 8% polyacrylamide gel (29:1 acrylamide: bisacrylamide) at 4°C using 1 $\times$ TBE running buffer. Gels were stained with 1 $\times$ NA-Red dye (Beyotime, #D0128) and visualized with a ChemiDoc MP (Bio-Rad) gel imager.

Optomotor response (OMR) assay

Visual acuity of mice was measured using an optomotor response-based (OMR) spatial frequency threshold test. Mice were allowed to move freely on a pedestal positioned at the center of a testing arena surrounded by four computer monitors arranged in a quadrangle. The monitors displayed a moving vertical black and white sinusoidal grating pattern with different spatial frequencies. During testing, a positive OMR was determined by repeated head angular movements in the direction of the black-white gratings [with different spatial frequency from 0.1-0.5 cycle/degree (cpd), at 100% contrast]. Head tracking was tested for both clockwise and counterclockwise directions, and each direction repeated four times. Three observers were 'blind' to mice groups to make a decision if mice have a positive OMR at the same time that mice received the gratings. Observers would make sure that the mouse do have an OMR under this spatial frequency when >50% positive OMR was judged by three observers from four times repeats, otherwise don't. Visual acuity was defined as the highest spatial frequency (at 100% contrast) yielding a threshold response.

Subretinal injection and sample collection

Subretinal injection of AAV vectors (packed by PackGene Biotech, Guangzhou, China) was performed at a titer of 1×10^{13} vg/mL, with a volume of 2 μ L per eye (equivalent to 2×10^{10} vg per eye). To minimize the impact of individual animal variability, the left eye of each mouse was injected with control AAV, while the right eye was injected with AAV carrying arRNA^B. Mice were anesthetized with intramuscular Zoletil 50 (50 mg/kg, 50 mg/mL), and both eyes were dilated with compound tropicamide eye drops. Periorbital skin was disinfected with povidone-iodine, and the bulbar conjunctiva was clamped and incised to expose the sclera. A coverslip was placed over the cornea (air bubbles eliminated with carbomer eye drops), and a disposable insulin needle was used to puncture the sclera and choroid to form a channel to the RPE-retina junction. The insulin needle was withdrawn, and a 33G microsyringe was inserted into the subretinal space via the channel for slow virus administration. The syringe was held in place for ~30 seconds to minimize leakage, and needles were cleaned to avoid cross-contamination. OCT scanning confirmed successful bilateral injection. Mice were observed twice daily for general condition throughout the experiment. On day 28, mice were euthanized by carotid artery exsanguination after anesthesia (per AVMA 2013 Guidelines). The retina-choroid-sclera complex was dissected for EGFP fluorescence examination under a stereomicroscope, or minced and homogenized in pre-chilled TRIzol (400 μ L) and stored at -70°C for RNA extraction.

Intravenous injection

Eight- to sixteen-week-old male and female AATD model mice weighing 18-26 g were used for intravenous AAV8 (experimental group) or PBS (control group) administration. Prior to injection, mice were gently restrained by hand to expose the lateral tail vein. A 30-gauge needle attached to a sterile syringe was used to puncture the lateral tail vein, and 200 μ L of the viral solution (1×10^{13} vg/mL) or PBS was slowly injected into the vein (ensuring no extravasation). Each mouse in the experimental group received a final viral dose of 2×10^{12} vg. After injection, the injection site was lightly pressed with sterile gauze for a few seconds to stop bleeding. Mice were returned to their cages and monitored briefly for abnormal reactions.

Library plasmid construction

The DNA oligonucleotide library with barcodes was synthesized by Twist Bioscience. Oligonucleotides were amplified using PhusionTM High-Fidelity PCR Master Mix (NEB, #M0531L) and primers containing the cleavage site of BsaI restriction enzyme. The PCR products were purified using DNA Clean & Concentrator-25 (Zymo Research, #D4034). Purified oligonucleotides were then ligated to the library plasmid backbone using Golden Gate assembly reaction (NEB, #E1602L). The reaction products were purified using DNA Clean & Concentrator-5 (Zymo Research, #D4014). Purified products were electroporated into *E. coli* HST08 Premium Electro-Cells (TAKARA, #9028) using Gene Pulser Xcell Microbial System (Bio-Rad, #1652662) to obtain the plasmid library.

A-C and A-U category dsRNA library screening

24 hours before transfection, 5×10^5 HEK293T cells were seeded in 6-well plates. The 293T cells used for transfection experiments were cultured in Dulbecco's modified Eagle's medium (Gibco, #C11965500BT) supplemented with 10% fetal bovine serum (Biological Industries, #04-010-1A) and without penicillin/streptomycin (P/S). 2 μ g of library plasmids were transfected into the cells according to the protocol of X-tremeGENE™ HP DNA Transfection Reagent (Roche, #6366546001). Cells were harvested 2 days after being transfected with the library plasmids. Total cellular RNAs were extracted using Quick-RNA™ MiniPrep (Zymo Research, #R1055). cDNAs were obtained by reverse transcription using HiScript® III 1st Strand cDNA Synthesis Kit (Vazyme, #R312). The primer for reverse transcription was Oligo-dT. cDNAs were amplified by PCR, and the PCR products (289 bp, containing barcodes) were used for next generation sequencing (Illumina HiSeq X Ten). The raw RNA-seq data generated in this study have been deposited in the National Genomics Data Center (NGDC, <https://ngdc.cnbc.ac.cn/>) under BioProject accession number PRJCA059931.

Western-blot assays

Soluble and insoluble protein were extracted in lysis buffer supplemented with a protease inhibitor cocktail (Sangon Biotech, #C500071) and total protein concentrations were quantified using a BCA assay. Lysates were then mixed with reducing SDS loading buffer, boiled, and separated by SDS-PAGE before being transferred onto PVDF membranes. Membranes were blocked for 60 min at room temperature and incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: anti-alpha antitrypsin (Abcam, #ab207303, clone EPR17087), anti- β -actin (Abcam, #ab6276), anti-ADAR1 (Cell Signaling Technology, #81284) and anti-GAPDH (EasyBio, #BE0024). After three washes with PBST, membranes were incubated with HRP-conjugated secondary antibodies for 60 min at room temperature. Protein signals were detected using an ECL substrate and visualized with a Chemidoc imaging system.

Immunofluorescence

Mouse eyes were collected immediately after euthanasia. The crystalline lens was segregated in PBS and the remaining eyecups were fixed in 4% paraformaldehyde (PFA) at 4°C for 24 h. Samples were embedded in optimal cutting temperature (OCT) compound, snap-frozen on dry ice, and stored at -80°C . Retina sections (10 μm thick) were prepared using a cryostat air-dried at 40°C for 30 min. After washing 3 times with PBS, sections were blocked with 1% BSA in 0.2% Triton X-100/PBS for 1 h at room temperature and incubated overnight at 4°C with primary antibody anti-USH2A (gifted from Leaper Bio Inc; 1:800 dilution).

Following three PBS washes, the retina sections were incubated with secondary antibody anti-rabbit IgG (H+L) (Thermo Fisher Scientific, #A11011) and CoraLite 488-Phalloidin (Proteintech, #PF00001; both at 1:500 dilution) for 2 h at room temperature. After three washes with PBST (0.2% Triton X-100 in PBS) and PBS, the retina sections were sealed with DAPI Fluoromount-G (SouthernBiotech, #0100-20). Images were acquired using a Zeiss LSM 980 laser confocal microscope, and subsequent image processing and analysis were performed using ZEISS ZEN Microscopy Software (Version 3.5.10).

Periodic Acid-Schiff with Diastase (PAS-D) Staining

Cut fresh mice liver tissue into small cubes. Transfer trimmed liver tissue to a labeled microcentrifuge tube filled with 4% PFA for fixation at 4°C overnight. Pre-heat the embedding station at 58°C . Transfer liver tissues into mold with paraffin covered bottom. Fill the mold to the top with molten paraffin, and transfer them to a cooling plate (4°C) for solidification. Liver paraffin sections (0.3 μm thick) were baked at 69°C for 1 h to enhance adhesion. Sequentially immerse in xylene (three times, 15 min each), 100% ethanol (15 min each), 95% ethanol (10 min), 80% ethanol (5 min), and ddH₂O (twice, 5 min each) to remove paraffin and rehydrate. Incubate rehydrated sections in Alpha-Amylase Solution (1%) for 20 min to hydrolyze glycogen. Wash sections with ddH₂O to terminate digestion (twice, 5 min each) to terminate digestion. Immerse sections in Periodic Acid Solution for 5 min. Wash sections with ddH₂O twice (twice, 5 min each) to remove excess periodic acid. Incubate sections in Schiff's Solution (dark, 15 min). Stain sections with Mayer's hematoxylin for 1 min. Wash sections with flowing water for 2 min and ddH₂O (twice, 5 min each). Treat sections with Blue Reagent for 5 seconds and washed with ddH₂O (twice, 5 min each). Related reagents for PAS-D staining are from Periodic Acid Schiff Diastase Stain Kit (Abcam, #AB287866). Dehydrate sections in 80% ethanol (5 min), 95% ethanol (5 min), 100% ethanol (twice, 5 min each), and xylene (twice, 5 min each). Mount with neutral balsam and cover with glass coverslips.

QUANTIFICATION AND STATISTICAL ANALYSIS

Structural prediction and interaction frequency analysis

The structural predictions for all ADAR1 and dsRNA complexes were conducted using the AlphaFold3 online server (<https://alphafoldserver.com/>). Protein-nucleic acid interactions were defined by two criteria: 1) atomic distances under 5 Å and 2) hydrogen bonding interactions, assessed via ChimeraX (<https://www.rbvi.ucsf.edu/chimerax>). We designated the central A-U/A-C pair in the deaminase-dsRNA interaction as the ADAR target site, shifting the site to the center of the sequence to create an interaction frequency heatmap centered on this target site. The pheatmap package (v1.0.12) generated the heatmap.

Structural analysis of RNA bulge effects on A-U/A-C categories

To create dsRNA sequences, we selected sequences interacting with the deaminase from the A-U library and generated the A-C category by substituting A-U target sites with A-C. For RNA bulges, a 5-nucleotide deletion was introduced in arRNA at position +2 and +11 (relative to the DRBMs direction and the A-U/A-C targeting site), producing a 5-nt bulge. These bulges were then incorporated into the previously mentioned precursor libraries, creating the final A-U/A-C library with RNA bulges.

RNA editing analysis

For assays analyzing fluorescence intensity, EGFP/mCherry/BFP-positive cells were sorted by flow cytometry (BD Fortessa SORP) 48 hours post-infection with mCherry-positive cells indicating successful expression of reporter genes, BFP-positive cells indicating successful transfection of arRNA and EGFP-positive cells indicate successfully edited cells. The percentage of fluorescent signal-positive cells was analyzed by FlowJo software (BD Biosciences). Control group was transfected with nonspecific arRNA of same length and same quantity as experimental groups.

For the experiments conducting NGS to measure exact editing rate, total RNA was extracted (Zymo Research, #R1055), reverse transcribed with HiScript® III 1st Strand cDNA Synthesis Kit (Vazyme, #R312), and PCR-amplified (TAKARA, #R051) using corresponding primers listed in supplementary files for further NGS analysis (Illumina HiSeq X Ten).

Analysis of library sequencing data

All the raw sequencing data was quantified with fastp by removal of low-quality reads before subsequent analyses with parameters: `-qualified_quality_phred 20 -length_required 50`. We calculated the editing levels in target sites using REDStat (<https://github.com/xuanxuan-Jin/REDStat>) which is a tool developed to investigate RNA editing level and patterns. During the calculating process, the barcodes with a coverage ≤ 50 reads were removed. The editing percentage was quantified as $G/(A + G) * 100$ at the target adenosine position. Data from different batches were using perfect double stranded (WT) sequence corrected.

Statistical analysis

Statistical tests and description of n were detailed described in every figure legend. Unless otherwise noted, n represents independent experiments performed in parallel (e.g., independent cell cultures, individual animal, etc.). The statistical significance was evaluated by one-way ANOVA, using GraphPad Prism software (GraphPad Software). Significance levels are indicated as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Supplemental figures

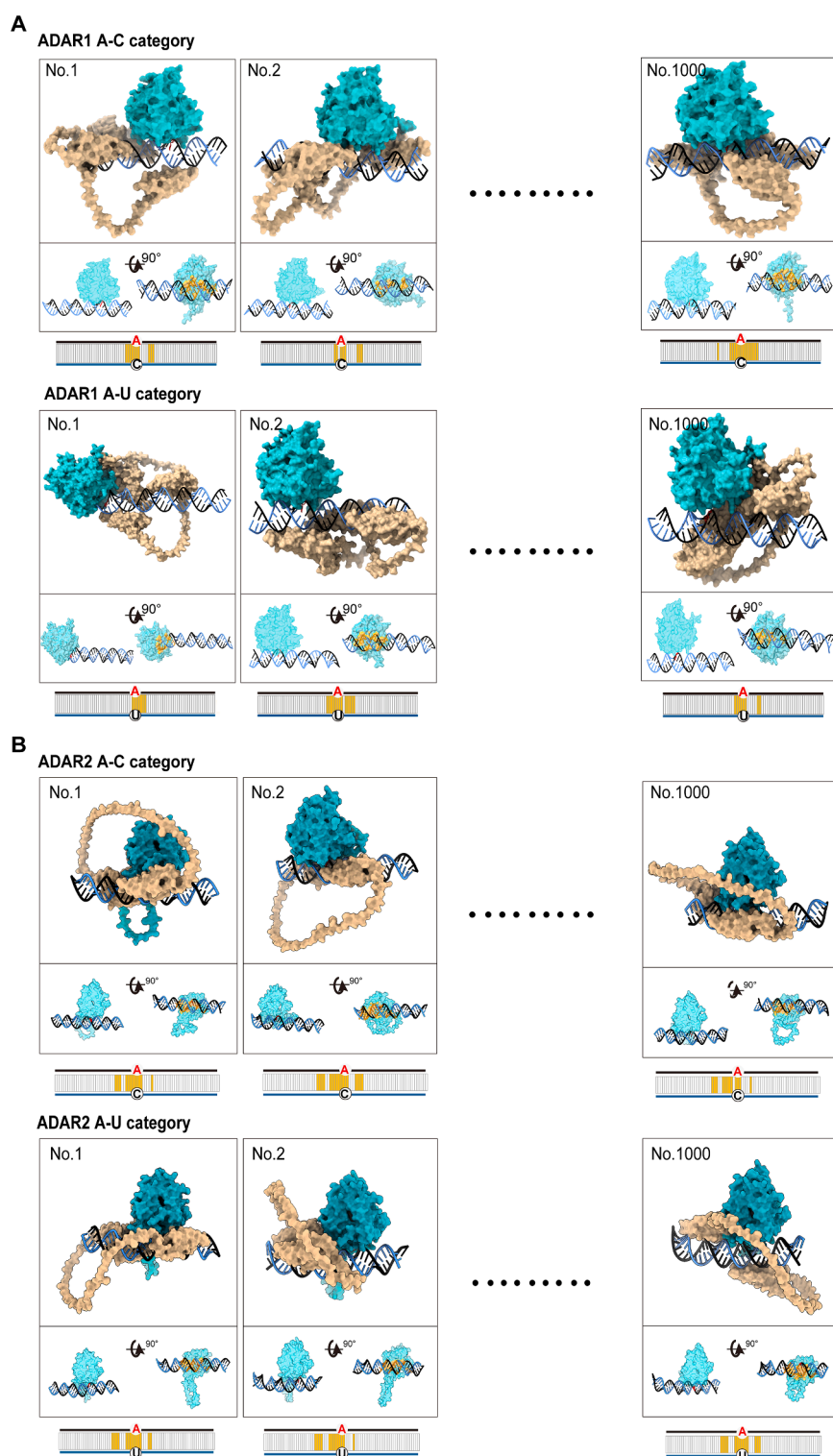


Figure S1. Workflow for analyzing deaminase-dsRNA interactions, related to [Figure 1](#)

Representative structural models of the ADAR1/2-dsRNA complex and the isolated deaminase-dsRNA subcomplex (with DRBM domains omitted), illustrating predicted interaction sites between the deaminase domain and dsRNA.

(A) A-C and A-U target categories for ADAR1 and (B) A-C and A-U target categories for ADAR2.

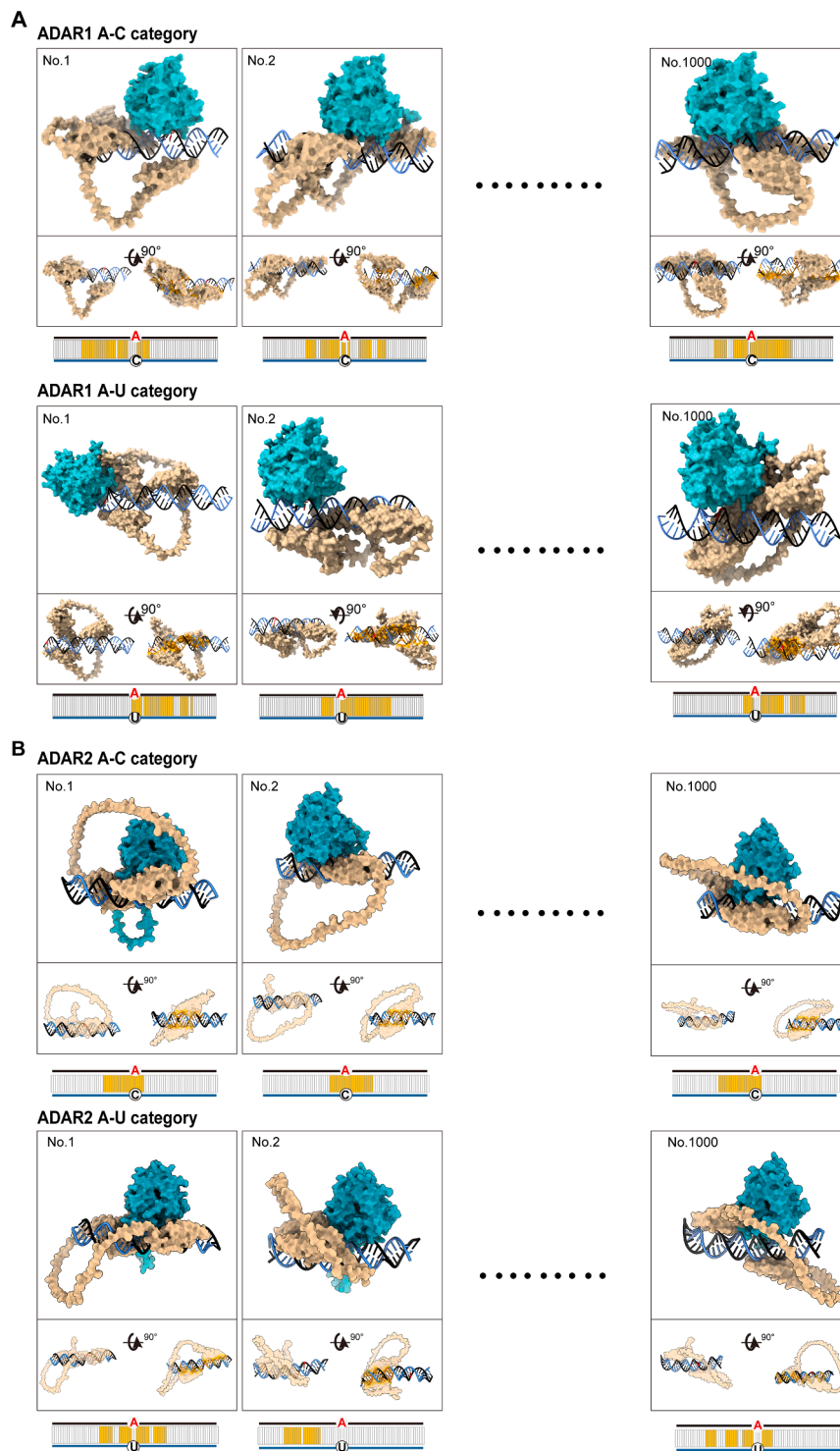


Figure S2. Workflow for analyzing DRBM-dsRNA interactions, related to Figure 1

Representative structural models of the ADAR1/2-dsRNA complex and the isolated DRBM-dsRNA subcomplex (with the deaminase domain omitted), highlighting predicted interaction sites between the DRBM domains and dsRNA.

(A) A-C and A-U target categories for ADAR1 and (B) A-C and A-U target categories for ADAR2.

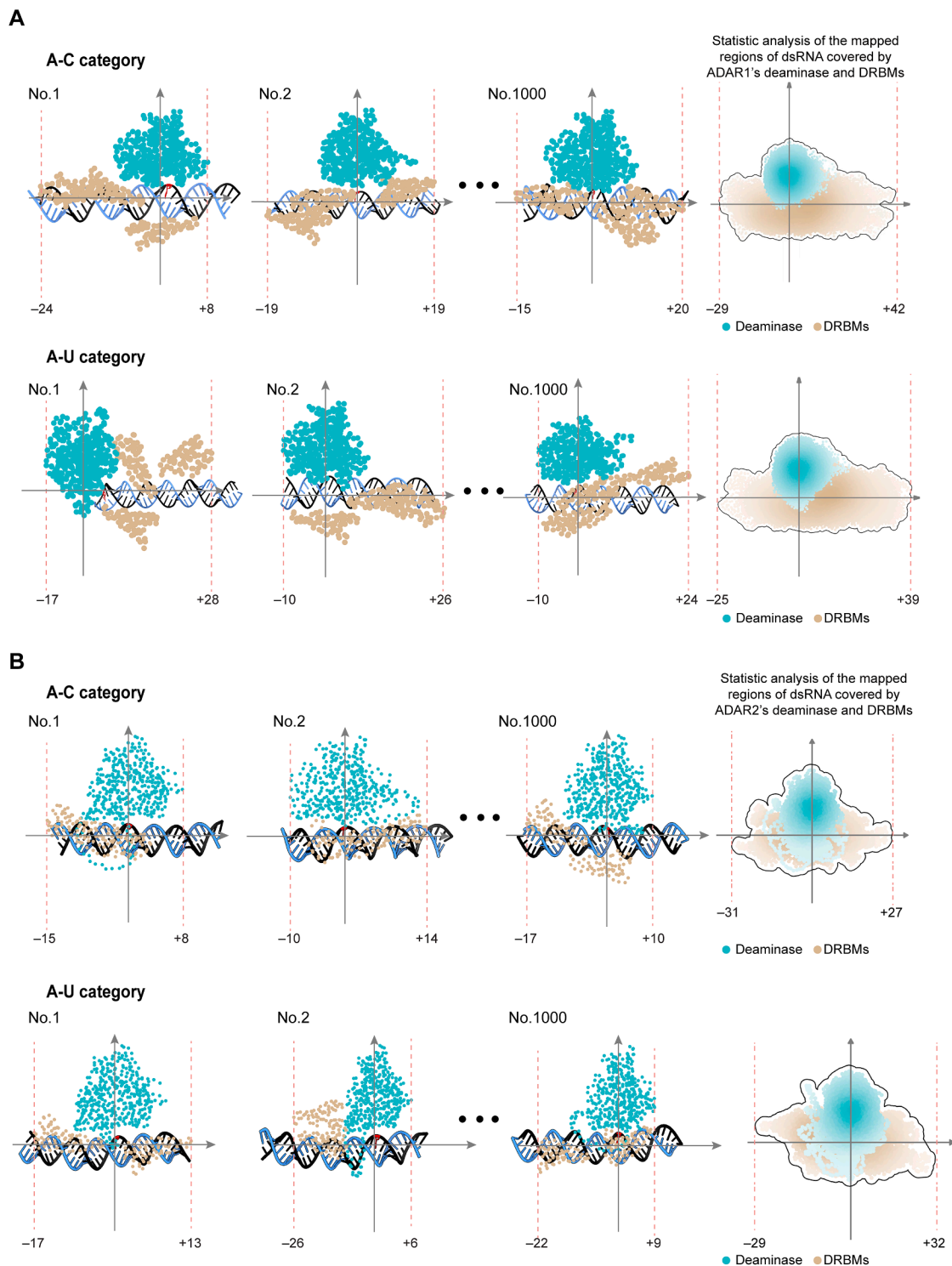


Figure S3. Overview of the ADAR-covered region analysis pipeline, related to Figure 1

Scatterplot analysis showing the distribution of C α atoms from ADAR1/2 deaminase and DRBM domains across predicted structures. Pink dashed lines indicate the boundaries of the ADAR-covered regions, with numeric labels denoting distances (in base pairs) relative to the target adenosine ("+" = 3' direction; "-" = 5' direction). An integrated heatmap summarizes C α positional data from all structural predictions, with color intensity representing positional density. (A) A-C and A-U target categories for ADAR1 and (B) A-C and A-U target categories for ADAR2.

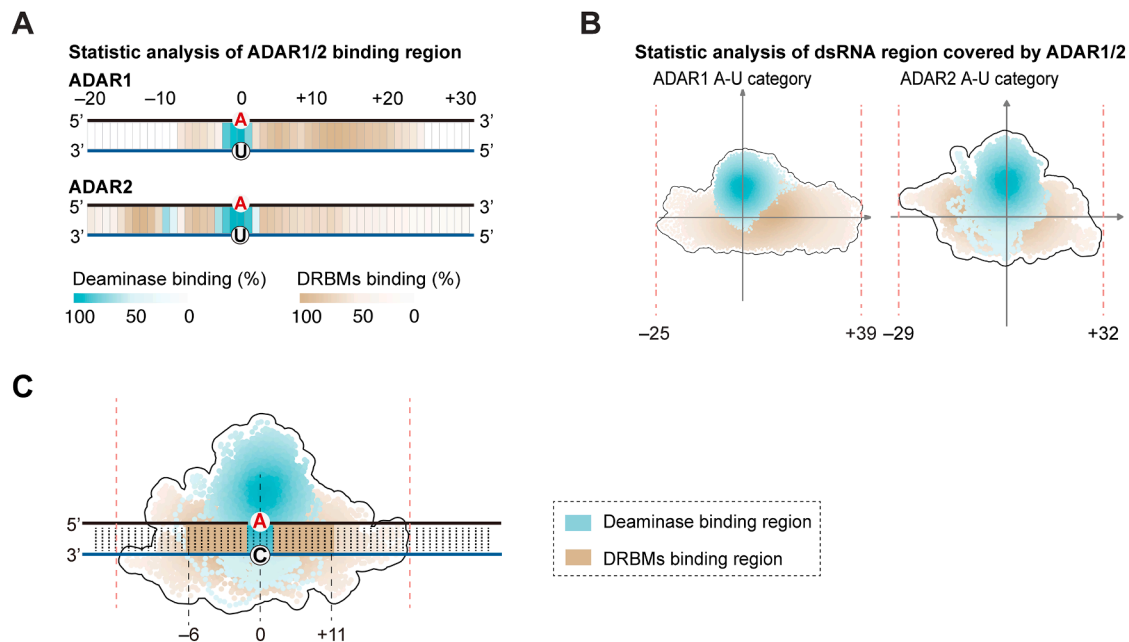


Figure S4. dsRNA regions engaged or covered by ADAR1 and ADAR2, related to Figure 1

(A) Heatmap depicting predicted ADAR1 and ADAR2 interaction sites on dsRNA containing A-U target sites, aggregated across all structural models.

(B) Density scatter plot illustrating the distribution of $C\alpha$ coordinates for the ADAR deaminase domain (blue) and DRBM domains (brown) relative to A-C target sites.

(C) Proposed structural model of ADAR2 binding to its dsRNA substrate.

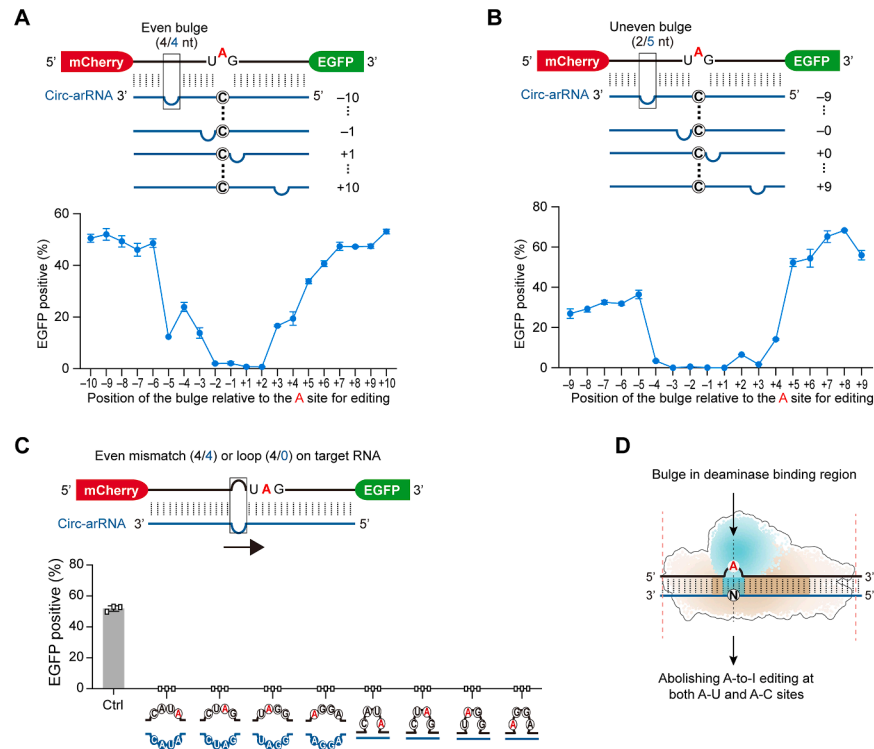


Figure S5. FACS analysis of EGFP-positive cells following transfection with arRNAs harboring bulges at different positions, related to Figure 2

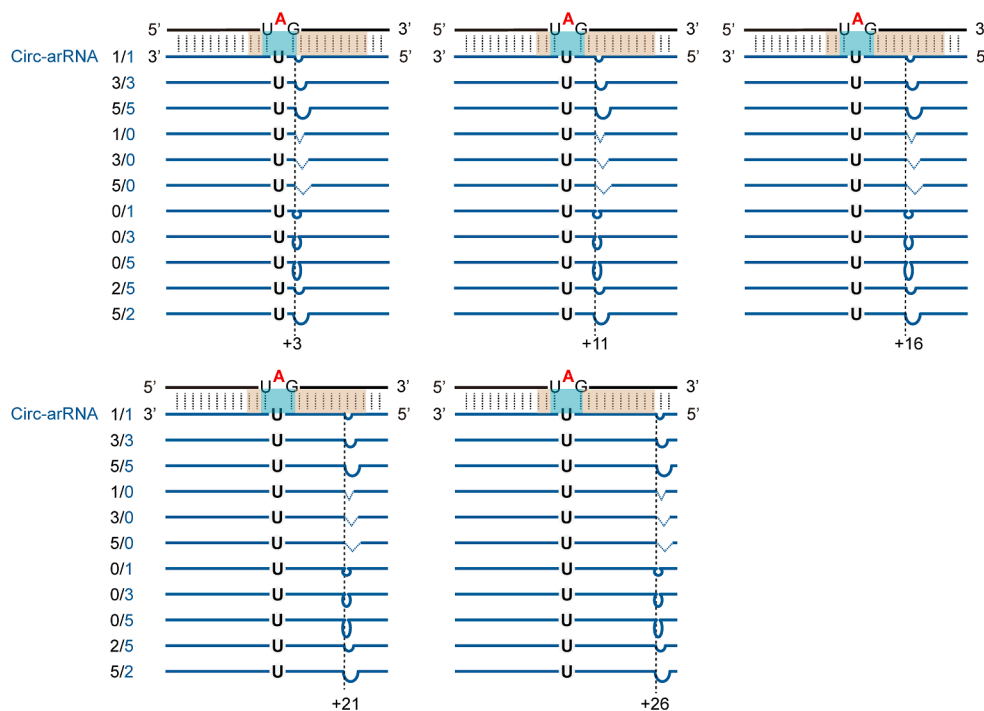
(A and B) Flow cytometry analysis showing the percentage of EGFP-positive cells after transfection with arRNAs containing either a 4/4 nt symmetric bulge (A) or a 2/5 nt asymmetric bulge (B) inserted at different distances from the target adenosine. A distinct reporter system was used compared with Figure 2. Data represent mean $\pm$ SD from three independent experiments ($n = 3$).

(C) FACS analysis showing EGFP-positive cells when the target adenosine is positioned directly within a 4/4 nt symmetric bulge or a 4/0 nt asymmetric bulge. Data represent mean $\pm$ SD from three independent experiments ($n = 3$).

(D) Schematic illustrating the introduction of RNA bulges into the deaminase-binding region.

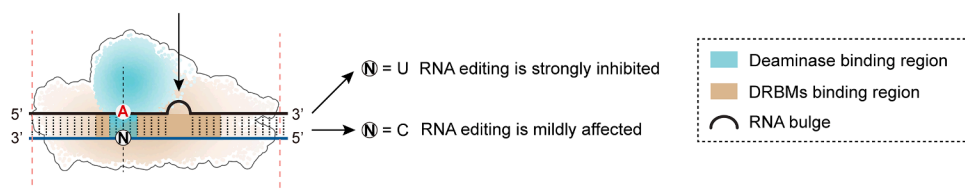
A

arRNA design of Figure 2D

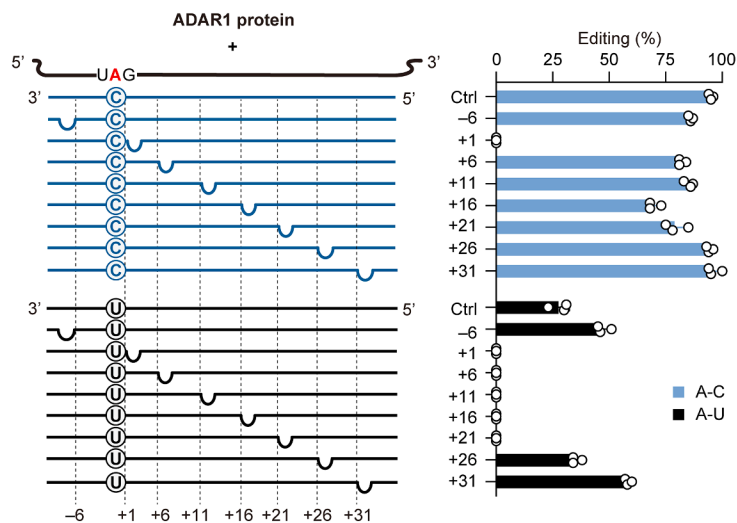


B

Bulge in DRBMs binding region



C



(legend on next page)

Figure S6. *In vitro* analysis of RNA bulge effects at A-U paired editing sites, related to Figure 2

(A) Schematic of the arRNA design used in the experiments shown in Figure 2D.

(B) Schematic illustrating the introduction of RNA bulges into the DRBM-binding region.

(C) Sanger sequencing results from *in vitro* deamination assays using purified ADAR1 p110. Target adenosines were designed as either A-C mismatches (blue) or A-U base pairs (black) depending on the arRNA construct. A-to-I editing efficiencies were quantified using EditR. Data represent mean $\pm$ SD from three independent experiments ($n = 3$).

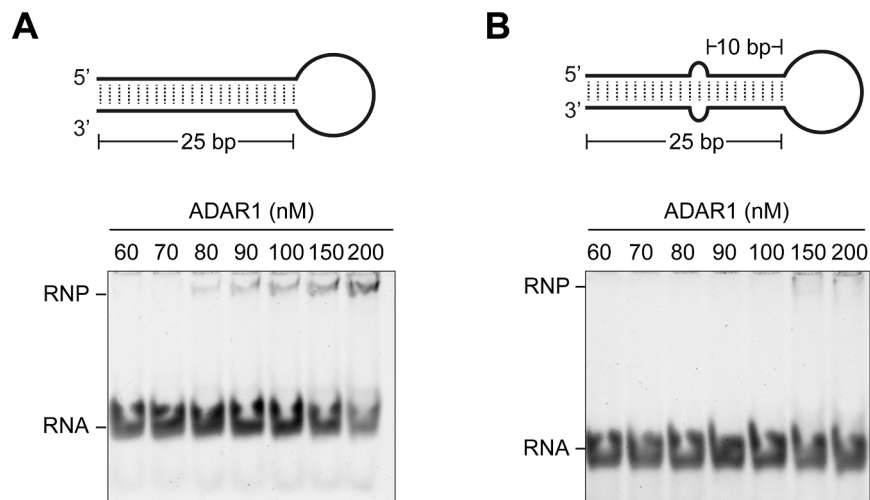


Figure S7. Electrophoretic mobility shift assays of dsRNA binding to ADAR1 p110, related to Figure 3

(A) EMSA of 25 bp dsRNA incubated with increasing concentrations of ADAR1 p110, demonstrating concentration-dependent binding.

(B) EMSA of 25 bp dsRNA containing a bulge, incubated with increasing concentrations of ADAR1 p110 to evaluate the effect of structural disruption on binding affinity.

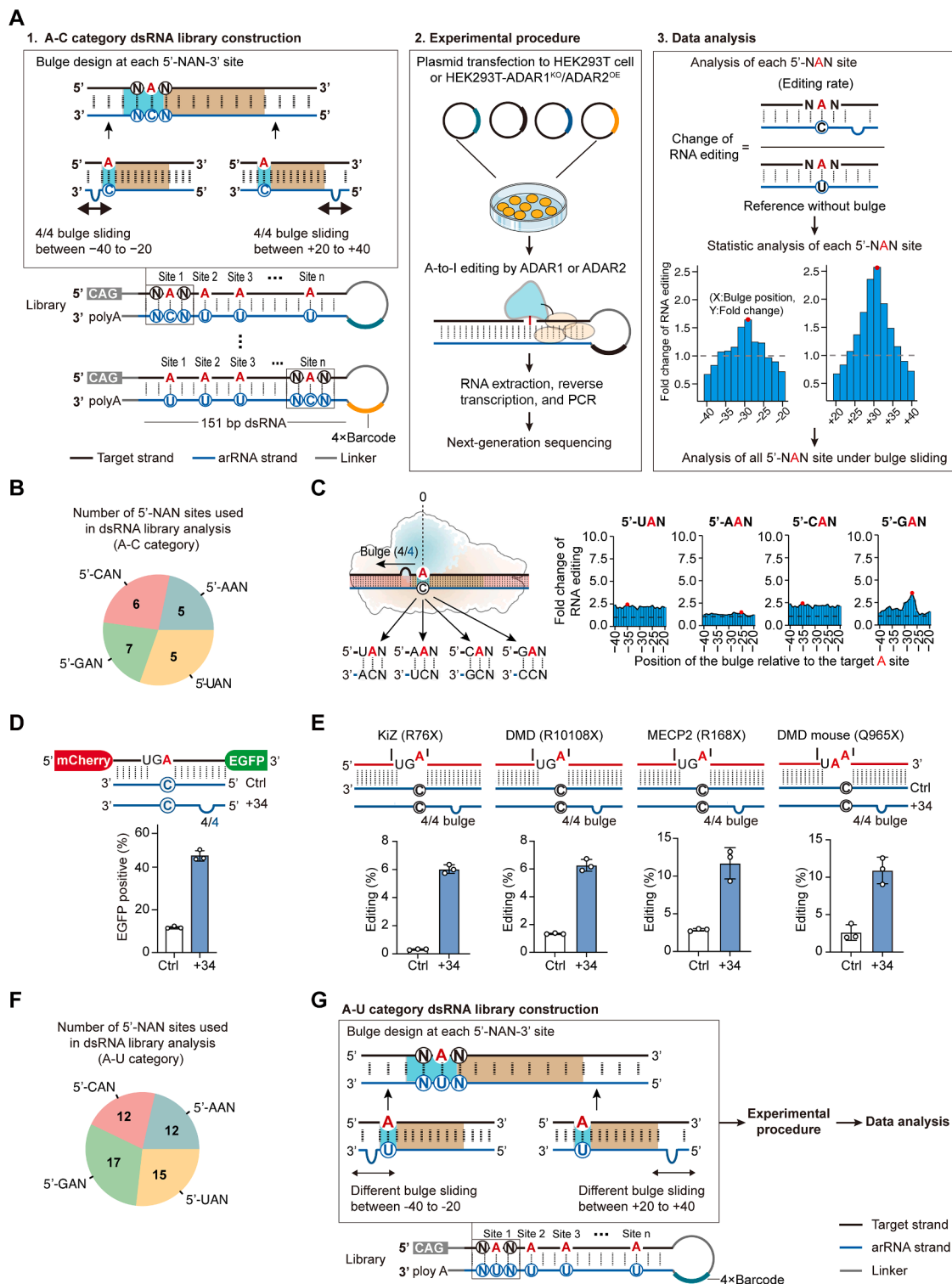


Figure S8. High-throughput screening of external RNA bulges using a dsRNA library approach, related to Figure 5

(A) Workflow of the bulge library screening. Left: schematic of the dsRNA library design, where each construct contains an A-C mismatched target site and a single 4/4 nt symmetric bulge positioned within the ADAR1/2-covered peripheral region (−20 to −2 and +20 to +40). The dsRNA duplex is generated from a single RNA transcript composed of a target region and arRNA region connected by a flexible linker containing a barcode. Transcription is driven by a CAG promoter and terminated with a poly(A) tail. Each sequence is associated with four unique barcodes to minimize noise from stochastic events.

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Middle: experimental workflow. The pooled library plasmid is transfected into HEK293T cells. Following RNA extraction, reverse transcription, and PCR, A-to-I editing efficiencies for each construct are quantified by NGS.

Right: data analysis pipeline. Editing rates at individual NAN motifs are extracted and normalized to a no-bulge reference control to calculate fold changes in editing efficiency.

(B) Pie chart showing the distribution of target-site types in the A-C mismatch library, categorized by the 5' base preceding the target adenosine.

(C) Fold-change analysis of editing efficiency following insertion of a 4/4 nt bulge at the 5' boundary of the ADAR1-dsRNA interaction region, categorized by 5' neighboring nucleotide context (UAN, AAN, CAN, GAN).

(D and E) Bar graphs showing enhanced editing efficiency in the LEAPER system for a reporter assay (D) and four disease-relevant targets (E) when arRNAs contain a 4/4 bulge at position +34, compared with bulge-free arRNAs.

(F) Pie chart showing the 5' base distribution of target adenosines in the A-U paired dsRNA library (analogous to B).

(G) Schematic of the A-U paired dsRNA library design, analogous to the A-C mismatch library described in (A).

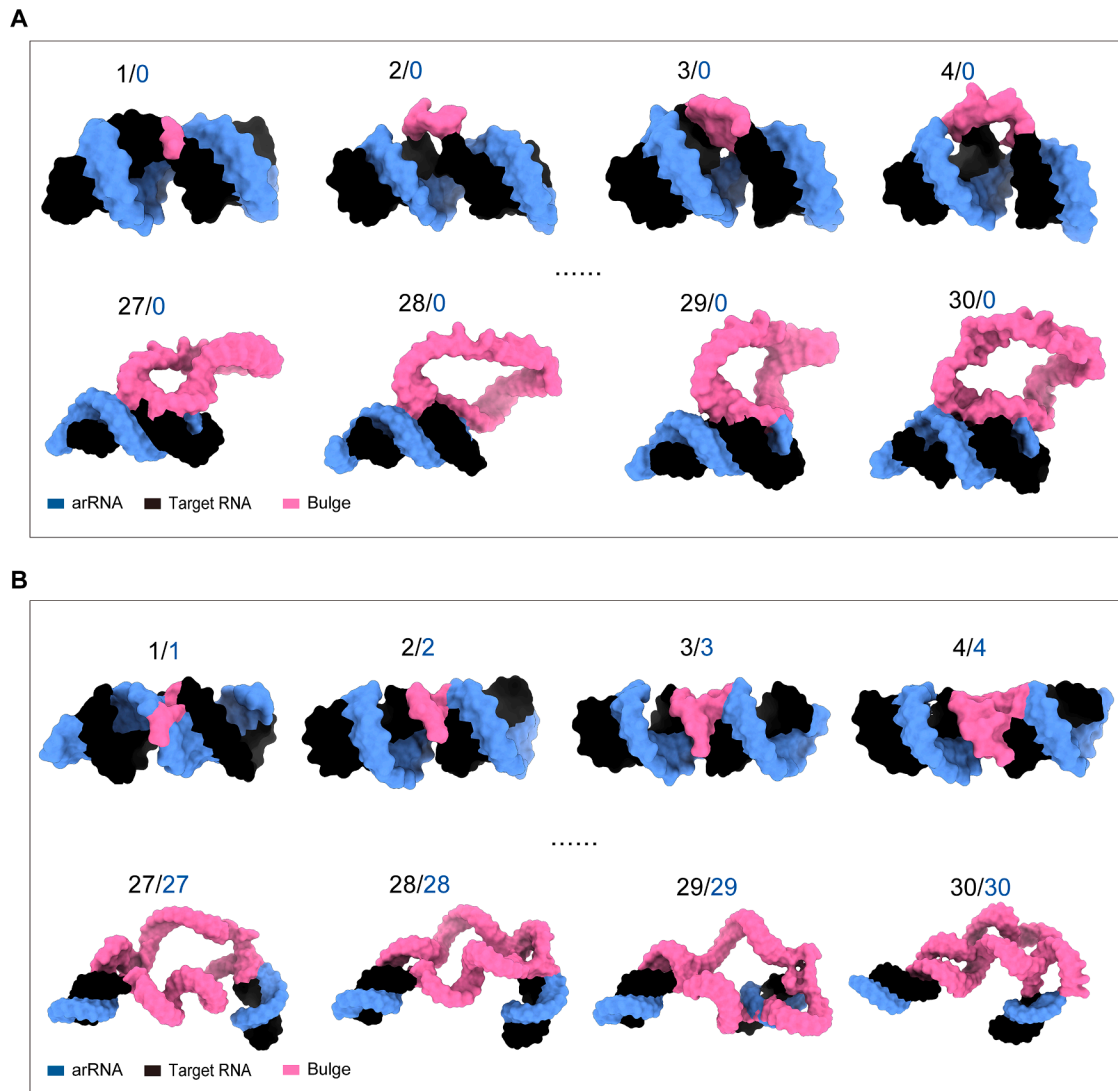
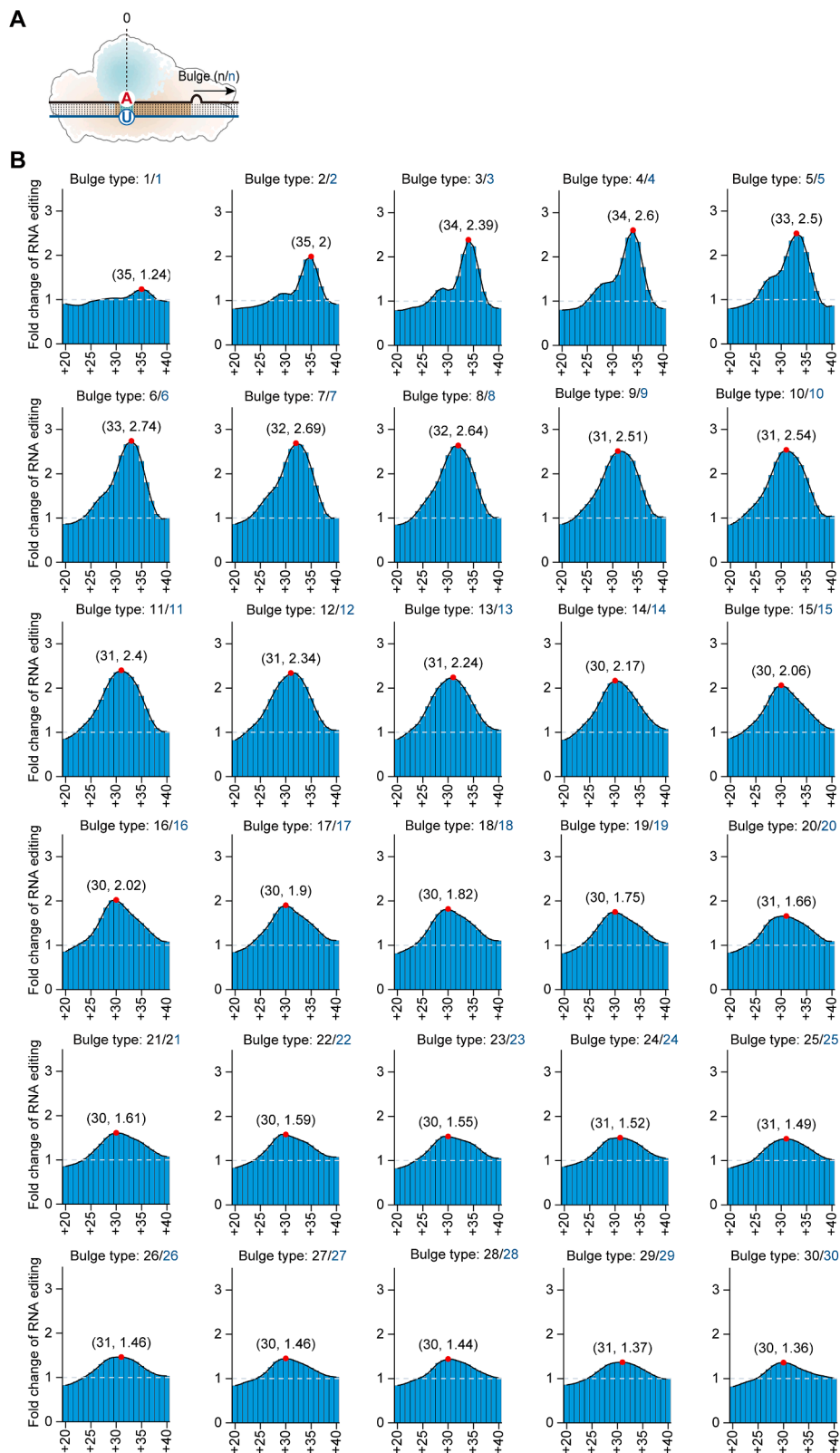


Figure S9. Structural visualization of RNA bulges with varying sizes and configurations, related to Figure 5

Predicted dsRNA structures containing bulges ranging from 1 to 30 nucleotides. The target RNA strand is shown in black, the arRNA strand in blue, and bulge regions are highlighted in pink.

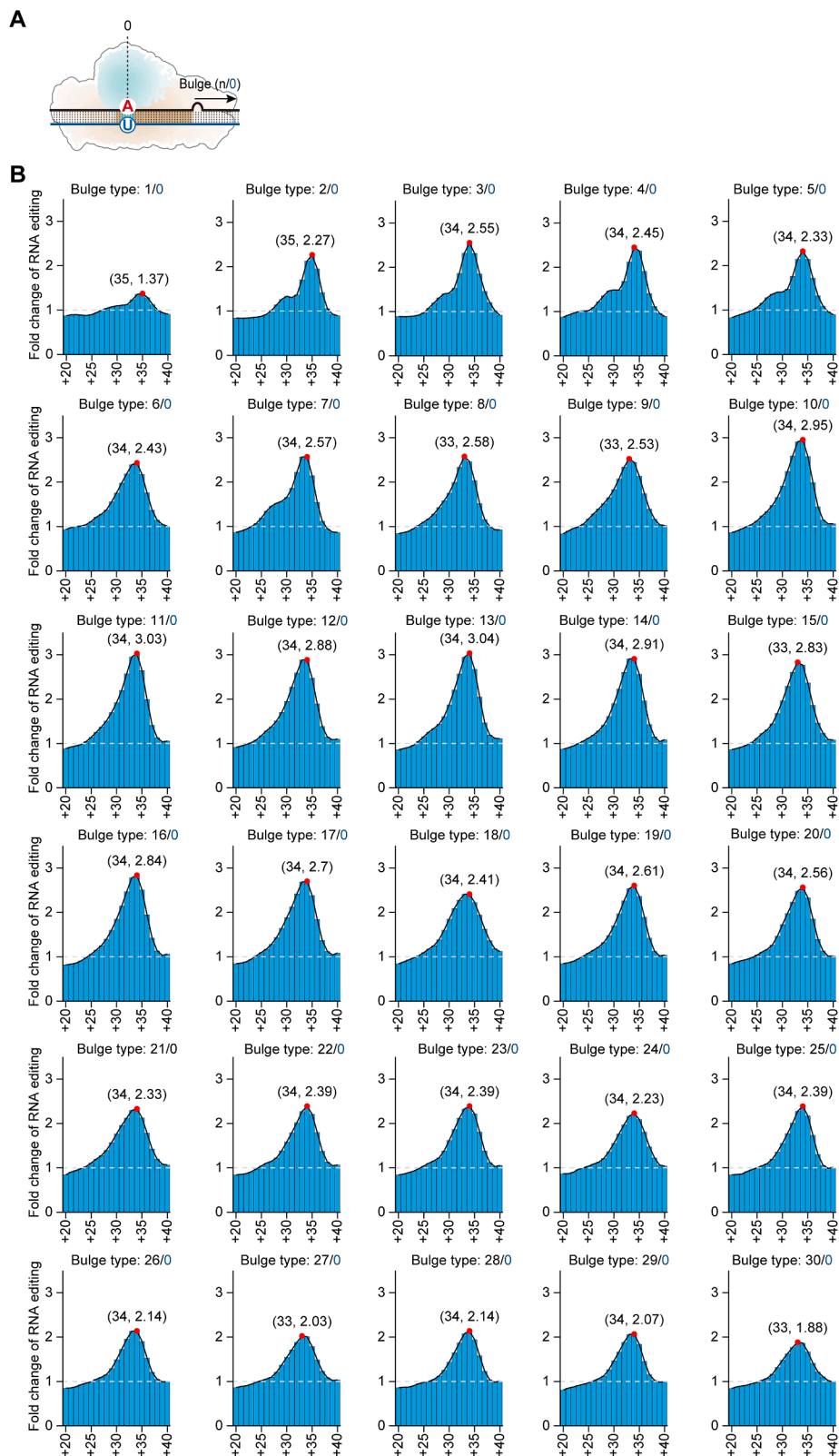
(A) n/0-type bulges (bulge on arRNA strand only) and (B) n/n-type bulges (bulges on both strands).



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Figure S10. A-to-I editing efficiency of arRNAs containing n/n-type bulges at different positions, related to [Figure 5](#)

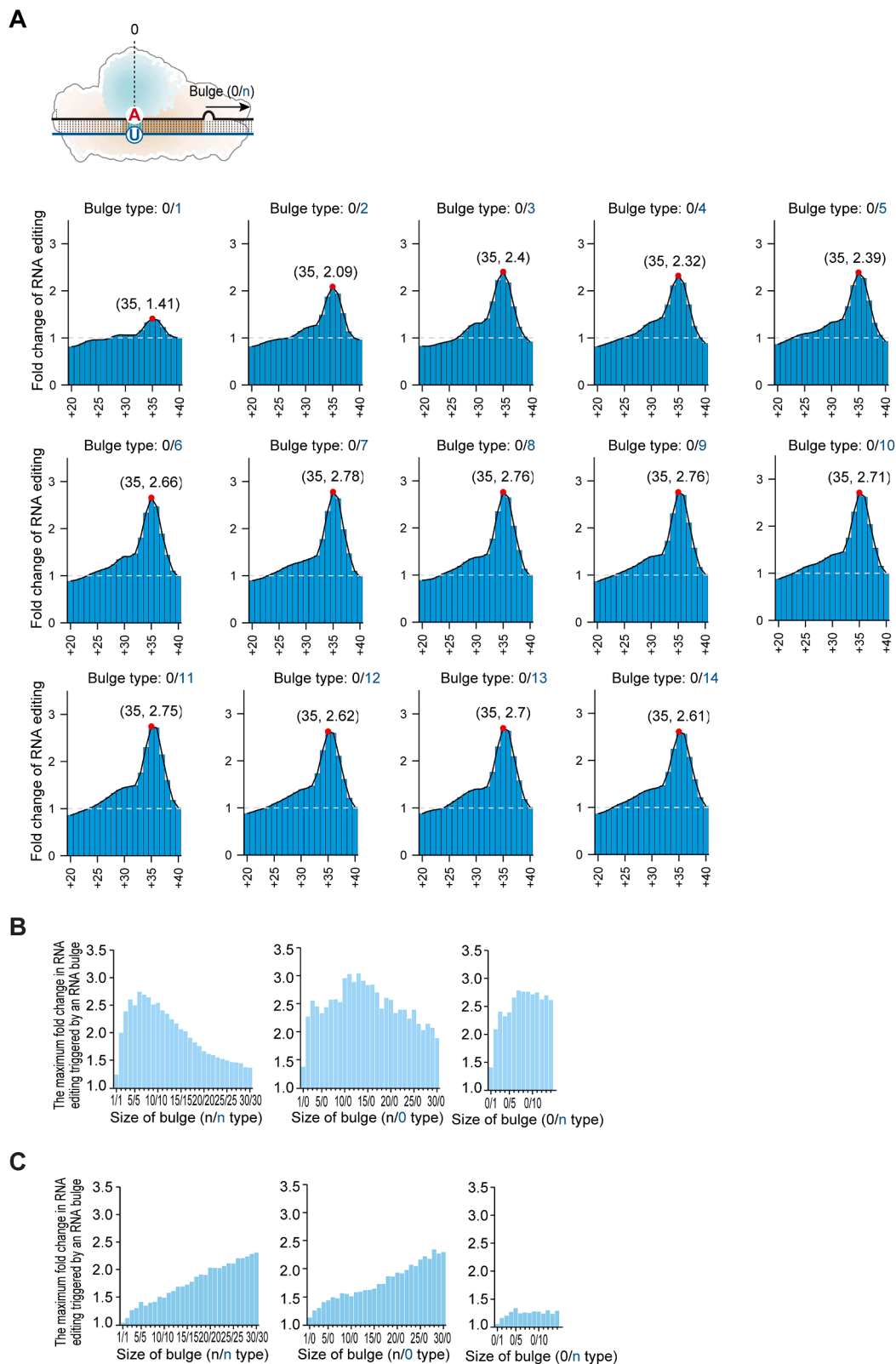
Bar graph showing editing efficiencies of arRNAs engineered with n/n-type bulges inserted at different positions along the arRNA-target RNA duplex, illustrating the positional effects of symmetric bulges on editing activity.



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Figure S11. A-to-I editing efficiency of arRNAs containing n/0-type bulges at different positions, related to [Figure 5](#)

Bar graph displaying editing efficiencies of arRNAs containing n/0-type bulges positioned at different locations along the arRNA-target RNA duplex, highlighting positional effects on RNA-editing efficiency.



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Figure S12. A-to-I editing efficiency of arRNAs containing 0/n-type bulges at different positions, related to [Figure 5](#)

(A) Bar plot illustrating editing efficiencies of arRNAs containing 0/n-type bulges at different positions along the arRNA-target RNA duplex, illustrating positional effects on ADAR1-mediated editing activity.

(B and C) Bar charts showing the maximum enhancement achieved by each bulge type (n/n, n/0, and 0/n) within the 3' ADAR1-covered peripheral region (C) or 5' ADAR2-covered peripheral region (D). Data were derived from library screens using A-U paired target sites.

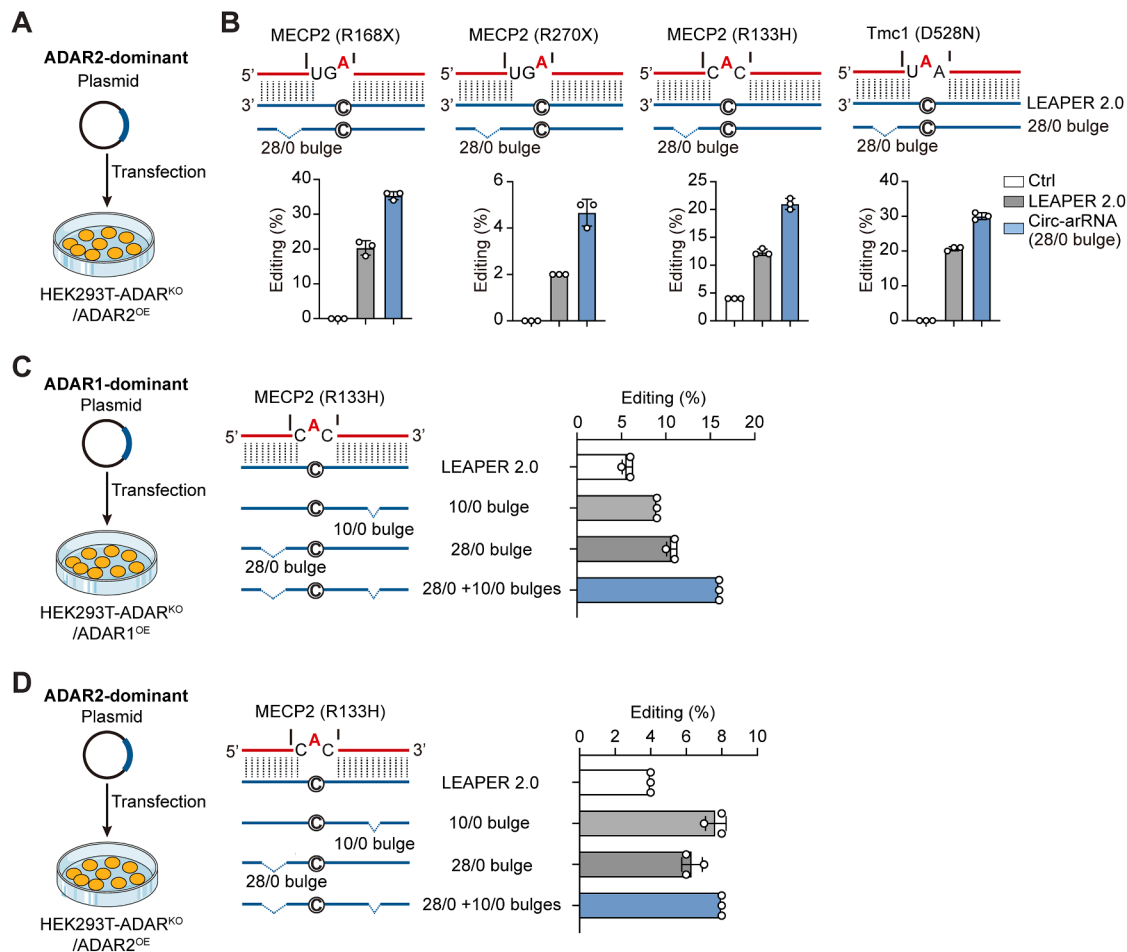


Figure S13. Optimization of arRNA bulge designs for RNA editing at disease-associated loci in ADAR1- or ADAR2-dominant cellular systems, related to Figure 5

(A) Workflow for evaluating arRNA-editing efficiency in an ADAR2-dominant cellular background, established by transfecting ADAR2 expression plasmids into ADAR-knockout HEK293T cells.

(B) Editing efficiency of arRNAs containing a 28/0 bulge at position -31 targeting four disease-relevant sites associated with Rett syndrome (*MECP2*) or congenital deafness (*Tmc1*).

(C) Experimental workflow and editing efficiency of arRNAs bearing single or dual external bulges targeting the *MECP2* R133H locus under ADAR1-dominant conditions.

(D) Experimental workflow and editing efficiency of arRNAs bearing single or dual external bulges targeting the *MECP2* R133H locus under ADAR2-dominant conditions.

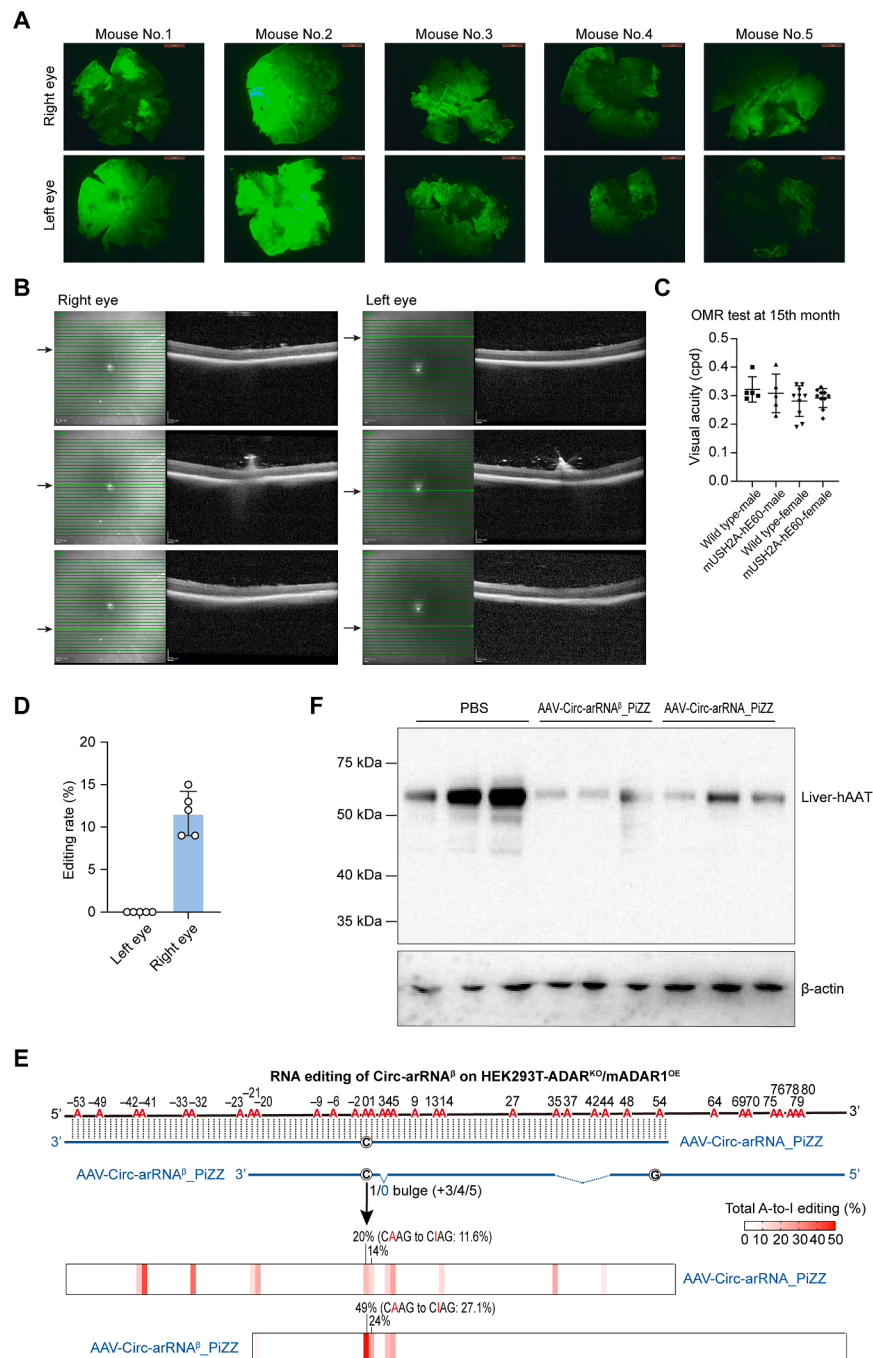


Figure S14. Additional analyses of AAV-delivered arRNA-mediated RNA editing in animal models and cellular assays, related to Figure 7

(A) Fluorescence images showing robust EGFP expression in retinal tissues following AAV8 delivery, indicating efficient infection and transgene expression. Retinas were subsequently harvested for RNA extraction and NGS analysis.

(B) *In vivo* optical coherence tomography (OCT) images showing preserved retinal integrity 28 days after injection. The characteristic hyperreflective signal corresponding to the optic disc is visible in the middle-layer images.

(C) Visual acuity (cycles per degree [cpd]) measured by optomotor response (OMR) in male and female wild-type C57 mice and mUSH2A-hE60 mice at 15 months of age. Data are shown as individual points with mean $\pm$ SEM, with each data point representing one mouse. No significant differences were observed between WT and humanized mice in either sex.

(D) *In vivo* A-to-I editing rates of the USH2A target site following treatment with arRNA^B or control AAV8 vectors at day 28 ($n = 5$).

(E) Heatmap showing A-to-I editing rates in HEK293T cells overexpressing mouse ADAR1, with precise editing rates for the target and bystander sites indicated.

(F) Western blot analysis showing human AAT protein levels in mouse liver following treatment with PBS (control) or arRNA vectors.

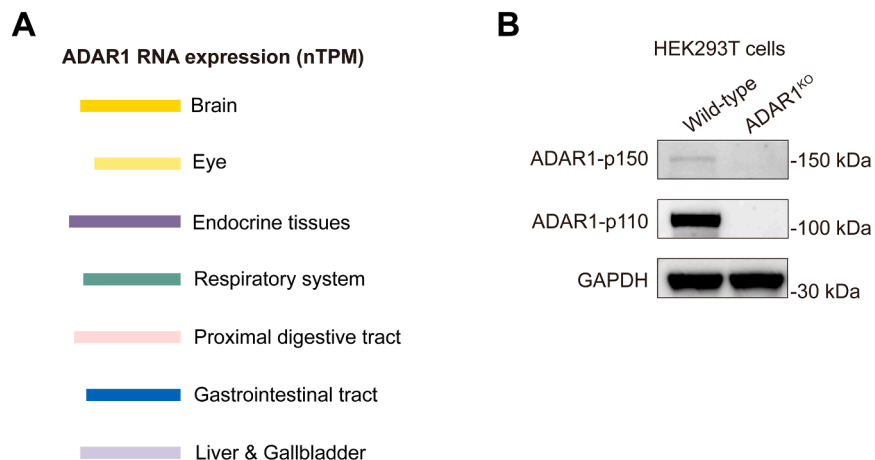


Figure S15. ADAR1 expression in HEK293T cells and in human and mouse tissues

(A) mRNA expression levels of ADAR1 normalized transcripts per million (nTPM) across human tissues based on data from the Human Protein Atlas (<https://www.proteinatlas.org>).

(B) Western blot analysis showing ADAR1 protein expression in wild-type HEK293T cells.