Chemically Modified AsCas12a Guide RNAs Improve Lipid Nanoparticle–Mediated *In Vivo* Gene Editing in Different Tissues

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INTRODUCTION

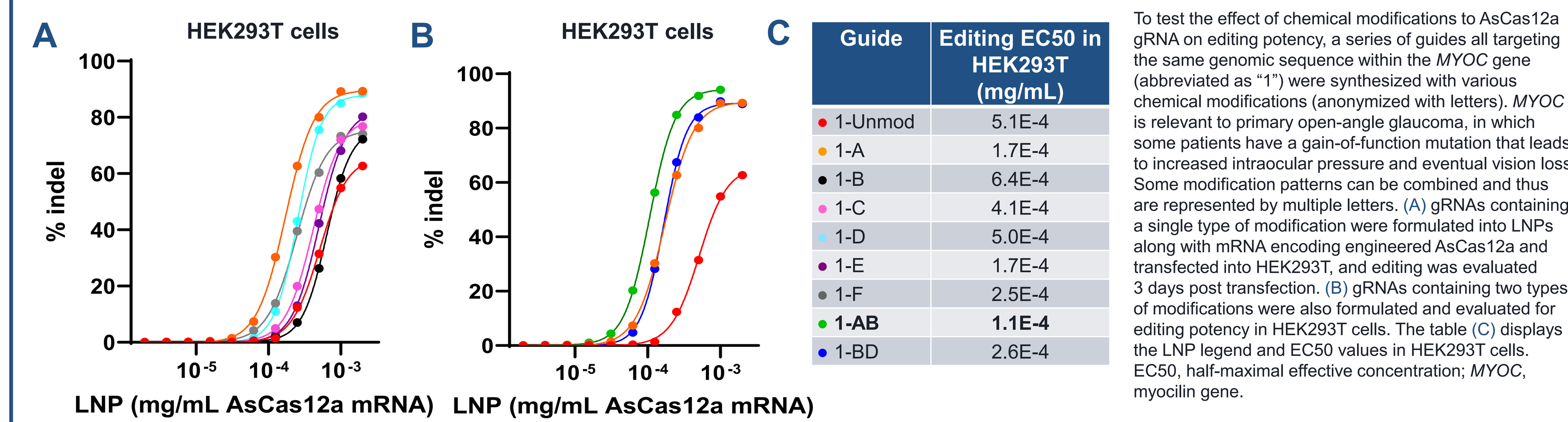
- Engineered *Acidaminococcus sp.* CRISPR-associated protein 12a (AsCas12a) is a potent and specific tool for gene editing.
- Lipid nanoparticles (LNPs) can deliver messenger RNA (mRNA) encoding AsCas12a nuclease and guide RNA (gRNA) to target cells *in vivo*.
- Nuclease digestion of gRNA in the cytoplasm limits editing efficiency, but chemical modification of gRNAs can protect them from degradation.
- The objective of this study was to evaluate chemical modifications of AsCas12a gRNA that would enable high-potency gene editing regardless of target cell type *in vitro* and *in vivo*.

METHODS

- **Guide synthesis:** gRNAs were synthesized via standard phosphoramidite chemistry. Purification was completed by ion-pair reversed-phase preparative high-performance liquid chromatography, followed by desalt and sequence analysis.
- **LNP formulation and quality control:** LNPs were formulated with commercially available lipids using a NanoAssemblr® Ignite™ (Cytiva). LNP cargo was mRNA encoding engineered AsCas12a nuclease and gRNA at a 1:1 ratio by weight. LNPs were evaluated for percent encapsulation greater than 80% by RiboGreen assay (ThermoFisher Scientific), polydispersity index (PDI) <0.2, and average diameter size <105 nm by Zetasizer analysis (Malvern Panalytical, Model ZSU3205).
- **Cell culture treatments:** Cells were treated with LNPs at indicated concentrations of encapsulated AsCas12a mRNA, and gDNA was isolated at 72 hours post transfection. Transfection of primary human hepatocytes (PHHs) included recombinant human apolipoprotein E. Amplicon-based next-generation sequencing (NGS) was performed to determine the percentage of editing.
- ***In vivo* editing in mouse eye:** LNPs were delivered into one eye of each $hMYOC^{Y437H}$ (human myocilin gene with the Y437H mutation) knock-in mouse via intracameral injection. One week post-injection, the eyes were dissected, and mRNA was isolated from the anterior chamber. A transcript-based RT-ddPCR assay was employed to measure the extent of remaining *hMYOC* mRNA.
- ***In vivo* editing in mouse liver:** LNPs were delivered via intravenous tail vein injection to $hMYOC^{Y437H}$ mice. One week post-injection, the livers were dissected, gDNA was isolated, and amplicon-based NGS was performed to determine the percentage of editing.
- ***In vitro* binding affinity measurements:** The labeled, unmodified guide was mixed with recombinant engineered AsCas12a and increasing concentrations of the modified "test" guide, incubated at room temperature for 3 hours, and then double filter separated on nitrocellulose blotting membrane (Cytiva) and Hybond N+ membrane (Cytiva). The fluorescently labeled, unmodified guide was quantified on each membrane and the percentage of bound unmodified guide was calculated. A decrease in the percent bound of the labeled, unmodified guide is a result of binding competition from the modified "test" guide.

RESULTS

Figure 1. Single and dual guide modifications improve *in vitro* editing



Guide	Editing EC50 in HEK293T (mg/mL)
1-Unmod	5.1E-4
1-A	1.7E-4
1-B	6.4E-4
1-C	4.1E-4
1-D	5.0E-4
1-E	1.7E-4
1-F	2.5E-4
1-AB	**1.1E-4**
1-BD	2.6E-4

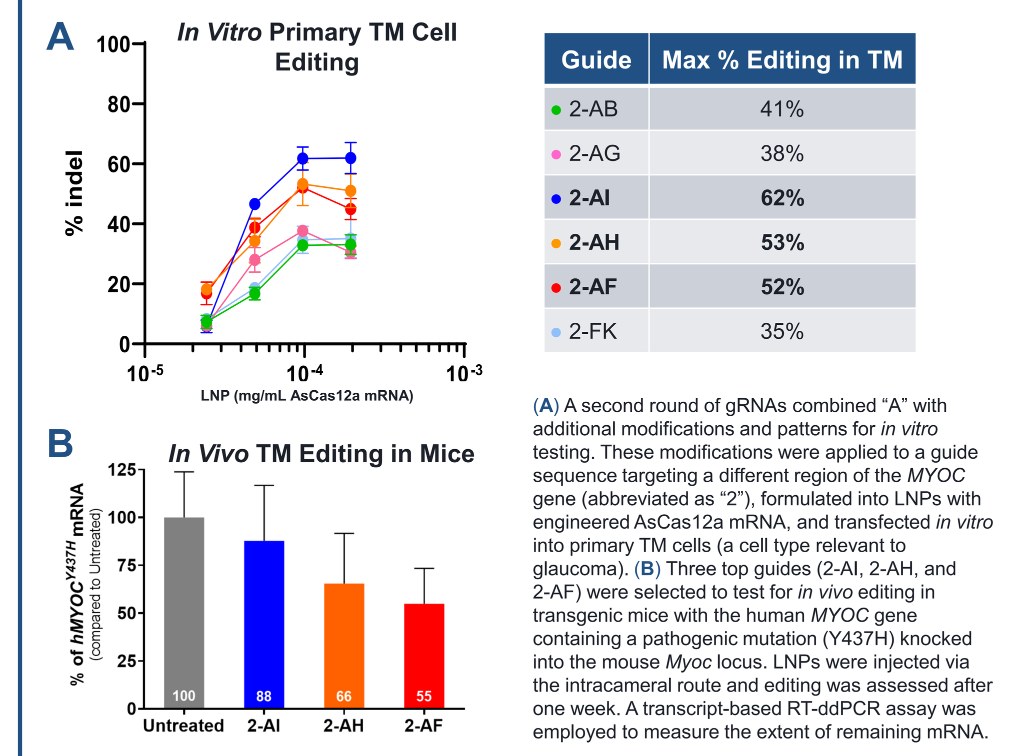
To test the effect of chemical modifications to AsCas12a gRNA on editing potency, a series of guides all targeting the same genomic sequence within the *MYOC* gene (abbreviated as "1") were synthesized with various chemical modifications (anonymized with letters). *MYOC* is relevant to primary open-angle glaucoma, in which some patients have a gain-of-function mutation that leads to increased intraocular pressure and eventual vision loss. Some modification patterns can be combined and thus are represented by multiple letters. (A) gRNAs containing a single type of modification were formulated into LNPs along with mRNA encoding engineered AsCas12a and transfected into HEK293T, and editing was evaluated 3 days post transfection. (B) gRNAs containing two types of modifications were also formulated and evaluated for editing potency in HEK293T cells. The table (C) displays the LNP legend and EC50 values in HEK293T cells. EC50, half-maximal effective concentration; *MYOC*, myocilin gene.

- Most of the modified gRNAs enabled efficient editing in HEK293T cells, and several different modifications improved the EC50 values, demonstrating improved editing potency compared to the unmodified guide.
- The "AB" combination pattern produced the most potent editing, and the "A" modification pattern is compatible with other types of modifications.

RESULTS continued

Figure 2. Combinations of gRNA modifications improve editing in trabecular meshwork (TM) cells *in vitro* and *in vivo*

- Combination of guide modification "A" with additional modifications and patterns led to improved editing potency in primary TM cells *in vitro*.
- Injection of LNPs containing the modified guides, especially 2-AF, resulted in a decrease in *MYOC* transcript, demonstrating that modified AsCas12a gRNAs enable *in vivo* gene editing in the mouse TM.

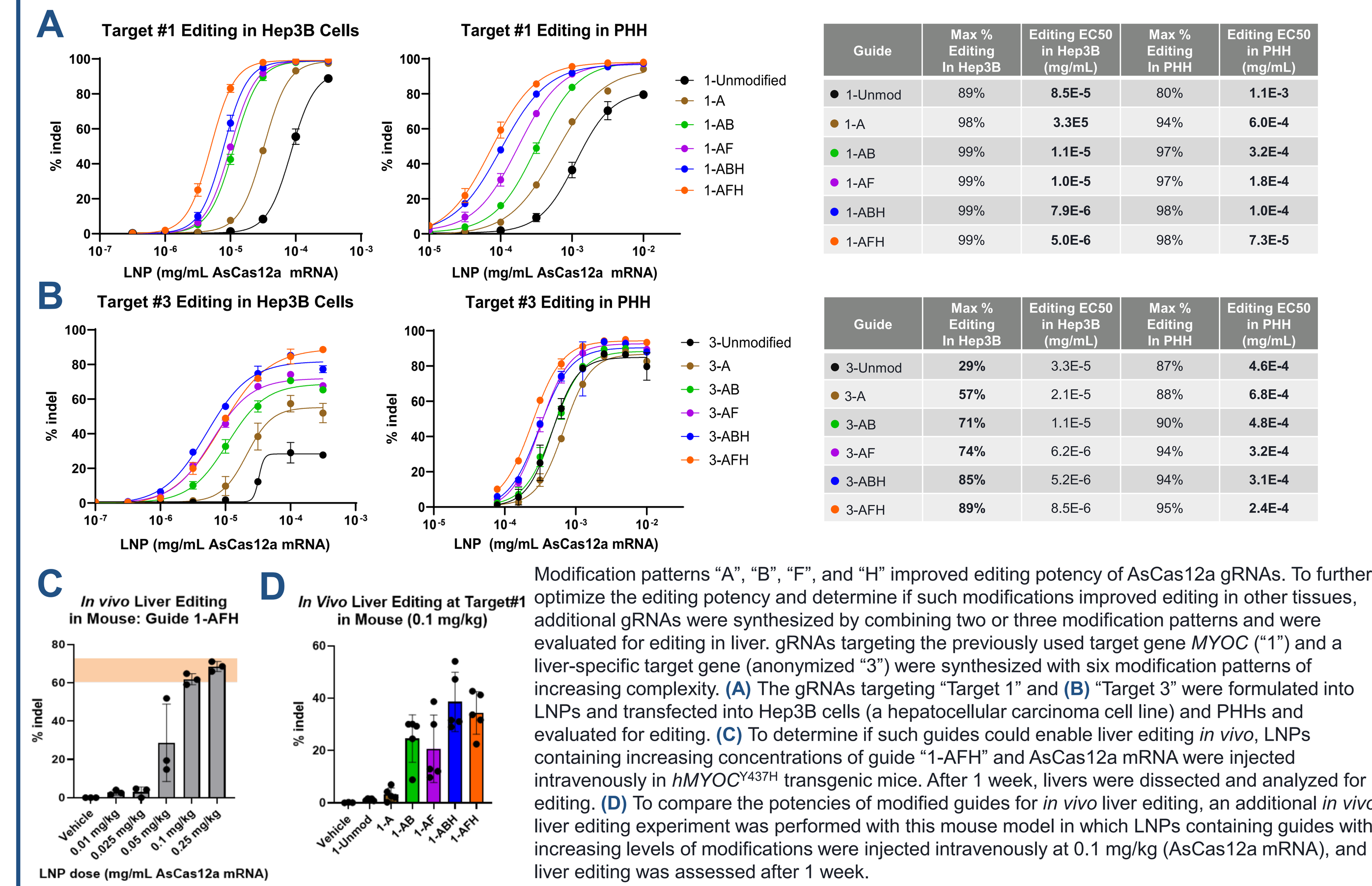


Guide	Max % Editing in TM
2-AB	41%
2-AG	38%
2-AI	**62%**
2-AH	**53%**
2-AF	**52%**
2-FK	35%

(A) A second round of gRNAs combined "A" with additional modifications and patterns for *in vitro* testing. These modifications were applied to a guide sequence targeting a different region of the *MYOC* gene (abbreviated as "2"), formulated into LNPs with engineered AsCas12a mRNA, and transfected *in vitro* into primary TM cells (a cell type relevant to glaucoma). (B) Three top guides (2-AI, 2-AH, and 2-AF) were selected to test for *in vivo* editing in transgenic mice with the human *MYOC* gene containing a pathogenic mutation (Y437H) knocked into the mouse *Myoc* locus. LNPs were injected via the intracameral route and editing was assessed after one week. A transcript-based RT-ddPCR assay was employed to measure the extent of remaining mRNA.

Figure 3. Additional combinations of gRNA modifications improve editing in liver cells *in vitro* and *in vivo*

- The "AFH" or "ABH" gRNAs produced the most potent editing *in vitro* in PHHs and Hep3B cells and *in vivo* in mouse liver for two separate target genes.
- At ≥0.1 mg/kg, "AFH" and "ABH" produced >60% liver editing *in vivo*. As mouse liver contains 60%–70% hepatocytes, >60% editing indicates that all or nearly all hepatocytes were edited.



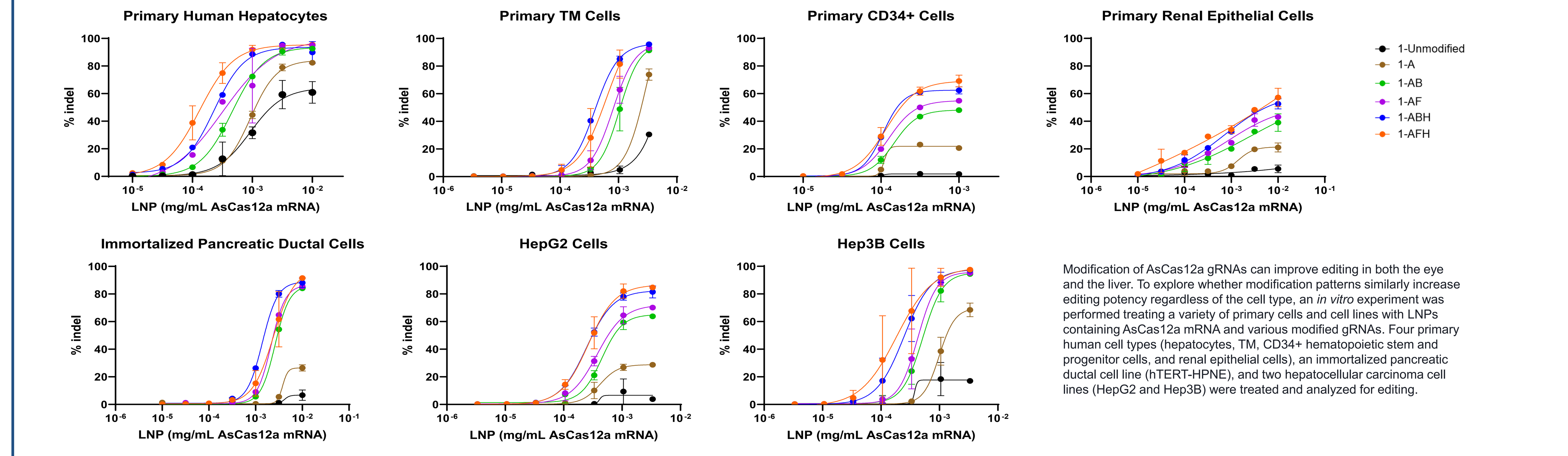
Guide	Max % Editing In Hep3B	Editing EC50 in Hep3B (mg/mL)	Max % Editing In PHH	Editing EC50 in PHH (mg/mL)
1-Unmod	89%	**8.5E-5**	80%	**1.1E-3**
1-A	98%	**3.3E5**	94%	**6.0E-4**
1-AB	99%	**1.1E-5**	97%	**3.2E-4**
1-AF	99%	**1.0E-5**	97%	**1.8E-4**
1-ABH	99%	**7.9E-6**	98%	**1.0E-4**
1-AFH	99%	**5.0E-6**	98%	**7.3E-5**

Guide	Max % Editing In Hep3B	Editing EC50 in Hep3B (mg/mL)	Max % Editing In PHH	Editing EC50 in PHH (mg/mL)
3-Unmod	**29%**	3.3E-5	87%	**4.6E-4**
3-A	**57%**	2.1E-5	88%	**6.8E-4**
3-AB	**71%**	1.1E-5	90%	**4.8E-4**
3-AF	**74%**	6.2E-6	94%	**3.2E-4**
3-ABH	**85%**	5.2E-6	94%	**3.1E-4**
3-AFH	**89%**	8.5E-6	95%	**2.4E-4**

Modification patterns "A", "B", "F", and "H" improved editing potency of AsCas12a gRNAs. To further optimize the editing potency and determine if such modifications improved editing in other tissues, additional gRNAs were synthesized by combining two or three modification patterns and were evaluated for editing in liver. gRNAs targeting the previously used target gene *MYOC* ("1") and a liver-specific target gene (anonymized "3") were synthesized with six modification patterns of increasing complexity. (A) The gRNAs targeting "Target 1" and (B) "Target 3" were formulated into LNPs and transfected into Hep3B cells (a hepatocellular carcinoma cell line) and PHHs and evaluated for editing. (C) To determine if such guides could enable liver editing *in vivo*, LNPs containing increasing concentrations of guide "1-AFH" and AsCas12a mRNA were injected intravenously in $hMYOC^{Y437H}$ transgenic mice. After 1 week, livers were dissected and analyzed for editing. (D) To compare the potencies of modified guides for *in vivo* liver editing, an additional *in vivo* liver editing experiment was performed with this mouse model in which LNPs containing guides with increasing levels of modifications were injected intravenously at 0.1 mg/kg (AsCas12a mRNA), and liver editing was assessed after 1 week.

Figure 4. gRNA modification patterns improve editing similarly regardless of cell type *in vitro*

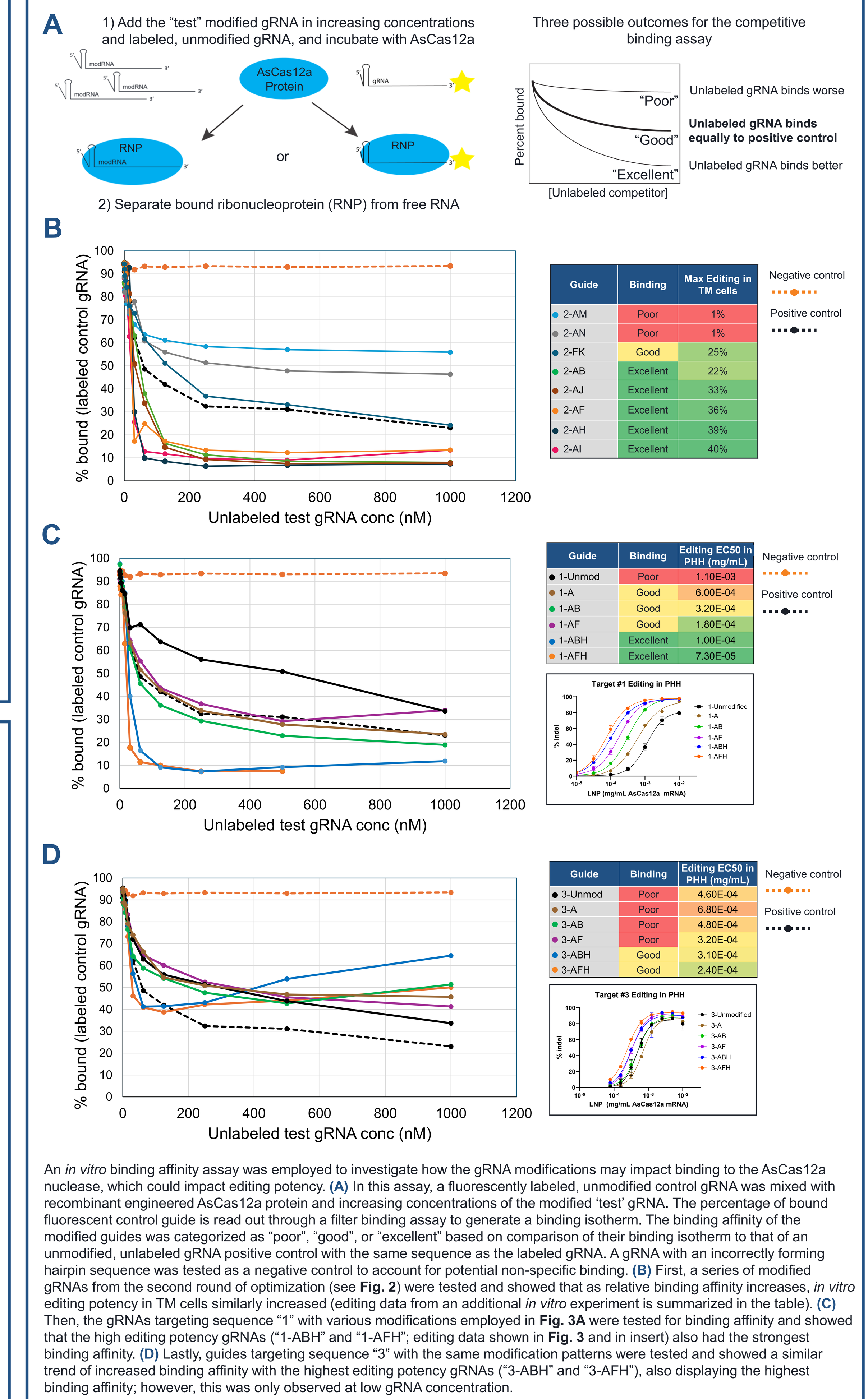
- Increased combinations of modifications led to improved editing potency across all cell types tested. gRNAs with the "AFH" and "ABH" modifications were the most potent, followed by the "AF" and "AB" modifications, followed by only "A". Unmodified gRNA was least potent.
- These results demonstrate the universality of the gRNA modification patterns for editing in many types of cells.



Modification of AsCas12a gRNAs can improve editing in both the eye and the liver. To explore whether modification patterns similarly increase editing potency regardless of the cell type, an *in vitro* experiment was performed treating a variety of primary cells and cell lines with LNPs containing AsCas12a mRNA and various modified gRNAs. Four primary human cell types (hepatocytes, TM, CD34+ hematopoietic stem and progenitor cells, and renal epithelial cells), an immortalized pancreatic ductal cell line (hTERT-HPNE), and two hepatocellular carcinoma cell lines (HepG2 and Hep3B) were treated and analyzed for editing.

Figure 5. Relative binding affinity correlates with editing potency

- gRNA modification patterns that increase relative binding affinity to the engineered AsCas12a nuclease also increase editing potency.



Guide	Binding	Max Editing in TM cells
2-AM	Poor	1%
2-AN	Poor	1%
2-FK	Good	25%
2-AB	Excellent	22%
2-AJ	Excellent	33%
2-AF	Excellent	36%
2-AH	Excellent	39%
2-AI	Excellent	40%

Guide	Binding	Editing EC50 in PHH (mg/mL)
1-Unmod	Poor	1.10E-03
1-A	Good	6.00E-04
1-AB	Good	3.20E-04
1-AF	Good	1.80E-04
1-ABH	Excellent	1.00E-04
1-AFH	Excellent	7.30E-05

Guide	Binding	Editing EC50 in PHH (mg/mL)
3-Unmod	Poor	4.60E-04
3-A	Poor	6.80E-04
3-AB	Poor	4.80E-04
3-AF	Poor	3.20E-04
3-ABH	Good	3.10E-04
3-AFH	Good	2.40E-04

An *in vitro* binding affinity assay was employed to investigate how the gRNA modifications may impact binding to the AsCas12a nuclease, which could impact editing potency. **(A)** In this assay, a fluorescently labeled, unmodified control gRNA was mixed with recombinant engineered AsCas12a protein and increasing concentrations of the modified 'test' gRNA. The percentage of bound fluorescent control guide is read out through a filter binding assay to generate a binding isotherm. The binding affinity of the modified guides was categorized as "poor", "good", or "excellent" based on comparison of their binding isotherm to that of an unmodified, unlabeled gRNA positive control with the same sequence as the labeled gRNA. A gRNA with an incorrectly forming hairpin sequence was tested as a negative control to account for potential non-specific binding. **(B)** First, a series of modified gRNAs from the second round of optimization (see **Fig. 2**) were tested and showed that as relative binding affinity increases, *in vitro* editing potency in TM cells similarly increased (editing data from an additional *in vitro* experiment is summarized in the table). **(C)** Then, the gRNAs targeting sequence "1" with various modifications employed in **Fig. 3A** were tested for binding affinity and showed that the high editing potency gRNAs ("1-ABH" and "1-AFH"; editing data shown in **Fig. 3** and in insert) also had the strongest binding affinity. **(D)** Lastly, guides targeting sequence "3" with the same modification patterns were tested and showed a similar trend of increased binding affinity with the highest editing potency gRNAs ("3-ABH" and "3-AFH"), also displaying the highest binding affinity; however, this was only observed at low gRNA concentration.

CONCLUSIONS

- Combinations of gRNA modifications improve editing potency *in vitro*, and the pattern of the gRNA modifications for improved editing is universal across a wide range of cell types.
- Combinations of gRNA modifications improved the potency of LNP-mediated gene editing by AsCas12a and gRNA *in vivo*.
- Increased editing potency of gRNA modifications is correlated with increased binding affinity of the gRNA with the engineered AsCas12a protein.

DISCLOSURES

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