In Vivo CRISPR Editing of Genetic Regulatory Regions Results in Functional Upregulation of Target Protein and Meaningful Reduction of Disease-Associated Biomarker in Mice

Benjamin Diner, Vikram Soman, Tushare Jinadasa, Ameya Apte, Meng Wu, Atti English, Steve Bottega, Mansi Thakkar, Deep Majithia, Luis Agosto, Shreya Jambardi, Emily Kaye, Briana Steward, James Bochicchio, Eugenio Marco, Tanya Teslovich, Linda Burkly, Jenny Xie, Paul Wrighton

Editas Medicine, Cambridge, MA, United States

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Figure 1: Editas' differentiated *in vivo* gene editing upregulation strategy can deliver first-to-market and best-in-class curative medicines for genetic diseases

Editing of gene regulatory regions, such as 5′ and 3′ untranslated regions, can induce upregulation of a wild-type or hypomorphic allele, functional homolog, or compensatory pathway to mitigate diseases caused by pathogenic, loss-of-function mutations:

	editas MEDICINE	Other Approaches	
Therapeutic Strategy	Functional Upregulation	Knockdown	Gene Correction
Gene Editing Approach	5′ Region or 3′ Region	Exon	Exon
Non–Gene Editing Modality	–	siRNA, ASO, monoclonal antibody, and small molecule	–
Patient Population	All patients (mutation agnostic)	All patients (mutation agnostic)	Subset of patients (single mutation)
Therapeutic Potential	First/Best-in class opportunities for loss-of-function diseases that cannot be addressed via knockdown	Diseases that can be addressed by protein reduction similar to ASO and siRNA	Correction limited to a subset of patients for a given disease

ASO, antisense oligonucleotide; siRNA, small interfering RNA.

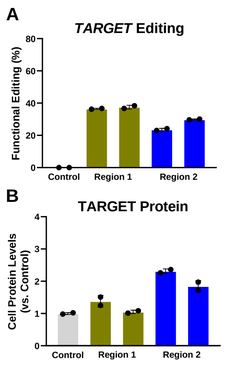
In order to treat sickle cell disease, we developed renizgamglogene autogedtemcel (reni-cel), a clustered regularly interspaced short palindromic repeats (CRISPR)–based therapeutic that upregulates γ-globin expression similarly to naturally-occurring, protective fetal hemoglobin (HbF)–inducing mutations found in individuals with hereditary persistence of fetal hemoglobin.

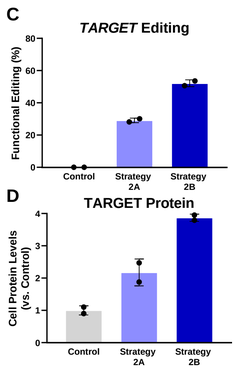
Here, we expand this validated upregulation strategy to develop a novel *in vivo* gene-editing medicine for an undisclosed **liver** indication based upon a naturally occurring, protective variant of the *TARGET* gene that results in its constitutive, elevated expression.

Figure 2: Editing of a *TARGET* regulatory region induces upregulation proof-of-concept in primary human hepatocytes and mice

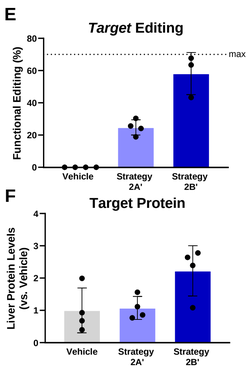
Based on the naturally occurring *TARGET* variant, we probed two purported gene regulatory regions for CRISPR-based upregulation:

RNA-LNP treatment of human hepatocyte culture





RNA-LNP treatment of wild-type mice



Editing *TARGET* regulatory Region 2 results in greater upregulation than Region 1 (A,B)

Optimizing the editing strategy for Region 2 improves TARGET protein upregulation (C,D)

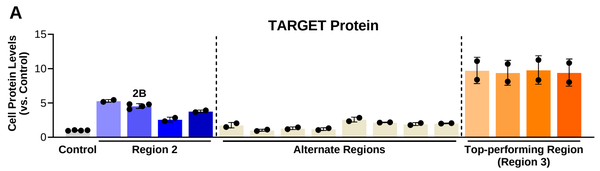
Editing of *Target* regulatory Region 2′ achieves upregulation *in vivo* (E,F)

Gene editing and subsequent upregulated expression of *TARGET* in human hepatocytes and livers of wild-type mice treated with standard liver-tropic lipid nanoparticles (LNPs) carrying Cas effector mRNA paired with various guide RNAs (gRNAs). Primary human hepatocytes were treated with RNA-LNPs and assessed for functional editing (those predicted to disrupt *TARGET* regulatory regions) by next-generation sequencing (NGS) **(A,C)** and lysate TARGET protein abundance by ELISA **(B,D)** at 4 days post-treatment (n=2). Wild-type C57BL/6 mice were dosed with a maximally-effective dose of RNA-LNP intravenously and assessed for functional editing and Target protein abundance in whole-liver homogenates **(E,F)** at 14 days post-treatment (n=4). " ' " denotes murine surrogate editing strategies homologous to the corresponding human-targeted strategy.

Figure 3: Comprehensive screening of regulatory regions delineated alternate editing strategies that further improve *TARGET* gene upregulation

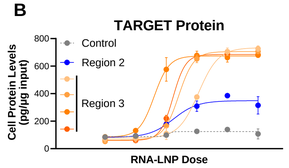
We sought to further optimize the CRISPR-based upregulation strategy by probing additional *TARGET* regulatory regions:

RNA-lipid treatment of human hepatocyte culture



Of the regulatory regions screened, disruption within Region 3 results in the largest induction of *TARGET* expression (A)

To identify the top-performing editing strategies for *TARGET* Region 3, we established their dose-response relationship as RNA-LNP:



C **Summary Table**

Strategy	Max Protein Upregulation (vs Control)	EC_{50}
Control	1	N/A
2B	2.9	$8.3x10^{-4}$
3A	6.8	$2.1x10^{-3}$
3B	6.6	$1.0x10^{-3}$
3C	6.3	$4.0x10^{-4}$
3D	6.4	$8.2x10^{-4}$

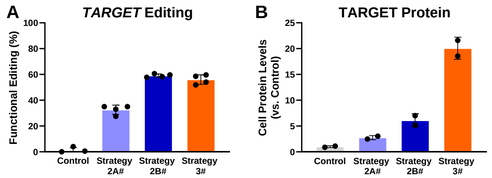
In RNA-LNP format, Region 3-disrupting strategies can be differentiated and all induce greater TARGET upregulation than Strategy 2B (B,C)

Comprehensive screen of variable CRISPR cargos directed against different *TARGET* gene regulatory regions-of-interest for their ability to induce *TARGET* expression in primary human hepatocytes **(A)**. Cultures were RNA-transfected at a maximally effective dose and TARGET abundances in lysates relative to a non-editing "Control" condition were measured by ELISA at 4 days post-transfection (n=2). RNA-LNP dose-response for select "Strategy 3" CRISPR system iterations in primary human hepatocytes **(B)**. Cultures were treated with variable RNA-LNP doses and TARGET abundance in lysates were measured by ELISA at 4 days post-transfection (n=2). Summary table displaying maximal observed TARGET protein fold-upregulation relative to a non-editing "Control" condition and half-maximal response RNA-LNP concentration (EC_{50}) **(C)**.

Figure 5: *TARGET* expression can be upregulated in non-human primate (NHP) hepatocytes using the same CRISPR-based strategies

We tested surrogate CRISPR strategies designed to disrupt NHP *TARGET* regulatory regions homologous to human Regions 2 and 3:

RNA-lipid treatment of NHP hepatocyte culture



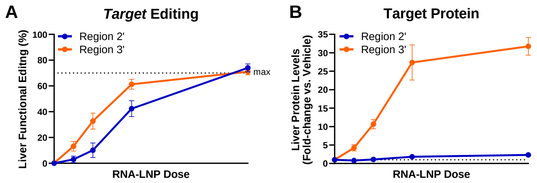
Editing homologous Region 3 induces greater *TARGET* expression relative to Region 2 in primary cynomolgus macaque hepatocytes (A,B)

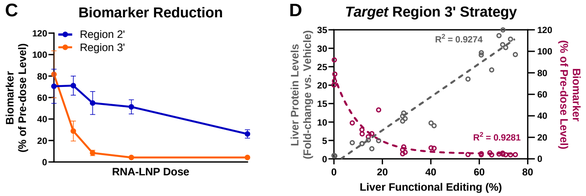
Gene editing and subsequent upregulated expression of *TARGET* in primary cynomolgus macaque hepatocyte culture treated with standard liver-tropic LNPs carrying Cas effector mRNA paired with various gRNAs. Hepatocytes were treated with RNA-LNPs and assessed for functional editing by NGS **(A)** and lysate TARGET protein abundance by ELISA **(B)** at 4 days post-treatment (n=2). TARGET protein abundances are expressed as values relative to non-editing "Control" conditions. " # " denotes primate surrogate editing strategies homologous to the corresponding human-targeted strategy.

Figure 4: *In vivo* editing of *Target* regulatory regions induces liver protein upregulation and reduction of a disease-associated biomarker in wild-type and disease model mice

We treated wild-type mice with LNPs containing surrogate cargoes targeting homologous murine regions Region 2′ and Region 3′:

RNA-LNP treatment of wild-type mice



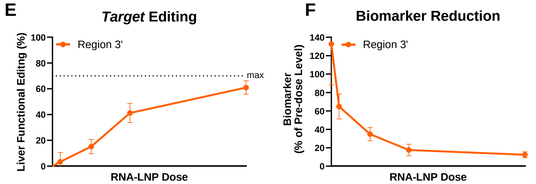


Robust editing of Region 3′ (A) **leads to greater Target protein upregulation** (B) **and biomarker reduction** (C) **than editing of Region 2′ *in vivo***

Functional editing of Region 3′ in the liver correlates strongly with Target protein upregulation and biomarker reduction in wild-type mice (D)

We treated mice harboring a disease-associated genetic variant phenocopying human disease with LNPs containing surrogate cargoes targeting homologous murine Region 3′:

RNA-LNP treatment of disease model mice



Functional editing of regulatory Region 3′ in the liver (E) **induces biomarker reduction** (F) **in a murine disease model**

In vivo assessment of liver editing, Target protein upregulation, and disease-associated biomarker abundance in mice. Wild-type or disease-associated genetic variant-harboring C57BL/6 mice were dosed with vehicle or standard liver tropic RNA-LNP intravenously and whole-liver homogenates were assessed for functional editing by NGS **(A,E)** and Target protein abundance by ELISA **(B)** at 14 days post-treatment (n = 6). Biomarker levels were measured at 13 days post-treatment and are expressed as % change relative to their levels at 2 days pre-treatment **(C,F)** (n = 6). Correlation analyses were performed to establish the relationship between functional liver genomic editing, liver Target protein abundance, and biomarker levels **(D)**. " ' " denotes murine surrogate editing strategies homologous to the corresponding human-targeted strategy.

Conclusions

- We established and optimized an *in vivo* gene upregulation strategy for an undisclosed indication based on a naturally-occurring, protective variant of the *TARGET* gene.
- Comprehensive *in vitro* screening delineated the most potent genetic regulatory region governing *TARGET* expression.
- Optimization resulted in lead editing strategies, which induce TARGET protein upregulation in primary human and cynomolgus macaque hepatocytes *in vitro.*
- An *in vivo* dose-response study demonstrated potent editing, Target protein upregulation, and meaningful reduction of a disease-specific biomarker in mice.

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