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Brief Report

Sequencing-guided design of genetically encoded small RNAs targeting CAG repeats for selective inhibition of mutant *huntingtin*

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Huntington's disease (HD) is an incurable neurodegenerative disorder caused by genetic expansion of a CAG repeat sequence in one allele of the *huntingtin* (*HTT*) gene. Reducing expression of the mutant HTT (mutHTT) protein has remained a clear therapeutic goal, but reduction of wild-type HTT (wtHTT) is undesirable, as it compromises gene function and potential therapeutic efficacy. One promising allele-selective approach involves targeting the CAG repeat expansion with steric binding small RNAs bearing central mismatches. However, successful genetic encoding requires consistent placement of mismatches to the target within the small RNA guide sequence, which involves 5′ processing precision by cellular enzymes. Here, we used small RNA sequencing (RNA-seq) to monitor the processing precision of a limited set of CAG repeat-targeted small RNAs expressed from multiple scaffold contexts. Small RNA-seq identified expression constructs with high-guide strand 5′ processing precision and promising allele-selective inhibition of mutHTT. Transcriptome-wide mRNA-seq also identified an allele-selective small RNA with a favorable off-target profile. These results support continued investigation and optimization of genetically encoded repeat-targeted small RNAs for allele-selective HD gene therapy and underscore the value of sequencing methods to balance specificity with allele selectivity during the design and selection process.

INTRODUCTION

Huntington's disease (HD), one of more than fifty known microsatellite repeat expansion disorders,[1] is caused by expansion of a CAG sequence to more than 37 tandem repeats in the first exon of a single *huntingtin* (*HTT*) allele.[2] The molecular pathology of HD is linked to the production of expanded polyglutamine tract-containing mutant HTT (mutHTT) protein, which confers gain-of-function as well as some loss-of-function pathology.[3–6] While the potential molecular and cellular disease mechanisms are diverse and remain to be fully characterized, safe and sustained reduction of mutHTT is expected to offer therapeutic benefit.[7] Two recent antisense oligonucleotide drug candidates targeting outside the repeat sequence, one against mutHTT-associated single nucleotide polymorphisms and one non-allele selective, did not yield positive results in patients.[8] While a number of factors will likely be important for clinical success, including patient stratification, approaches that preserve wild-type HTT (wtHTT), while selectively reducing mutHTT continue to be preferred.[4,8,9]

Allele-selective mutHTT reduction has been previously demonstrated with ASOs[10–12] as well as artificial microRNAs (miRNAs)[13–17] and small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) with central mismatches,[11,17,18] delivered both virally and non-virally. Proposed mechanisms involve binding CAG repeats to preferentially block translation or induce degradation of mRNAs containing CAG repeat expansions[10,14,17,19] (Figure 1A). For repeat expansion selectivity with small RNAs, miRNA-like binding rather than cleavage by Argonaute 2 (Ago2) must be achieved, which is accomplished via central mismatches between the small RNA and target.[14,15,17,18] When genetically encoded, this strategy requires precise processing of repeat RNA stem-loops that are typically prone to slippage during folding.[18,20–22]

Here, we investigated the cellular processing, allele selectivity, and off-target effects of genetically encoded small RNAs with central mismatches against the CAG repeat expansion of mutHTT mRNA. We evaluated a limited set of small RNA expression scaffolds with different stem structures, loop sequences, and central mismatch

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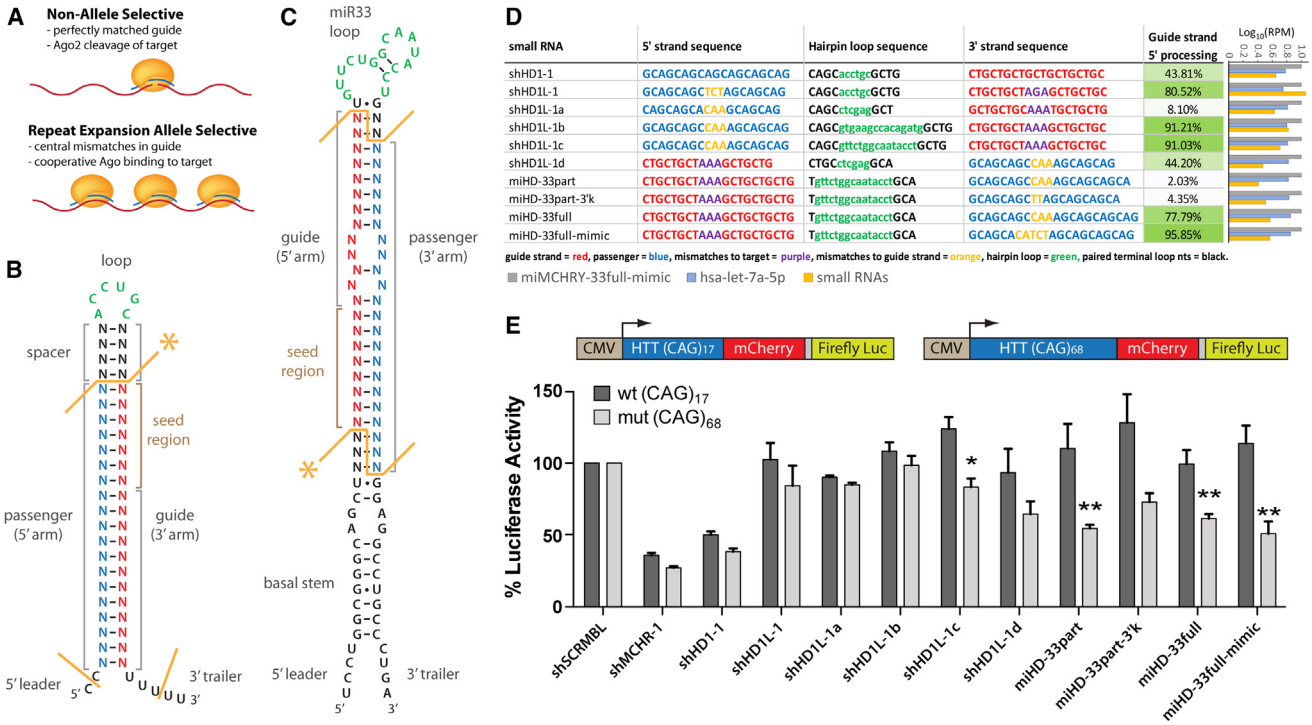


Figure 1. Design and function of CAG repeat-targeting small RNAs

(A) Illustration of the principle of allele-selective targeting of repetitive RNA sequence by small RNAs that bind but do not cleave target RNA. (B and C) (B) Design of an shRNA scaffold and (C) a miRNA scaffold for expression of small RNAs targeting CAG repeats. Key elements of the design are indicated. Predicted cleavage sites generated during processing are indicated with orange lines. Asterisks indicate predicted 5′ processing of the guide strand. (D) DNA-level sequence of the 5′ arm, hairpin loop, and 3′ arm of small RNA expression constructs. The 5′ guide strand processing precision, which is critical for proper mismatch placement when paired with the mRNA, and relative abundance (plot to the right) normalized to a control miMCHRY-33full-mimic small RNA construct (expressing non-targeting mCherry small RNA) are reported based on small sequencing results after transient transfection of expression constructs in HEK 293T cells. (E) Activity of wt $(CAG)_{17}$ or mut $(CAG)_{68}$ repeat-containing luciferase reporters in HEK 293T cells after transient transfection with small RNA expression vectors. Error bars are standard deviation. *$p \leq 0.05$, **$p < 0.01$, as compared with wt $(CAG)_{17}$.

sequences. High 5′ processing precision of guide strands and, therefore, consistent positioning of central mismatches, was observed for several small RNAs using sequencing. Multiple designs exhibited selectivity for CAG expansions or mutHTT in model cells or patient-derived cells when encoded into lentiviral or adeno-associated viral (AAV) vectors. Endogenous miRNA profiles and transcriptome-wide gene expression were monitored for a select set of small RNAs, which revealed varying degrees of potential off-target effects, including for other CAG repeat-containing mRNAs,[18,23] as well as one small RNA with a favorable off-target profile. These results should contribute to the ongoing development of safe, allele-selective small RNAs that can be vectorized for potential gene therapy of HD. They also demonstrate the value of sequencing to guide the design and selection of genetically encoded small RNAs where precise processing and robust knockdown must be balanced with high specificity.

RESULTS

Design and processing of CAG repeat-targeted small RNA expression vectors

To generate small RNA expression vectors, we cloned small RNA duplexes (Table S1) into a U6 promoter-driven lentiviral vector expressing a separate EGFP-puromycin reporter[24,25] (Figure S1A). Small RNAs were expressed from two core constructs, an shRNA[24] (Figure 1B) or a miRNA (Figure 1C) scaffold.[20] The shRNA scaffold typically expresses guide strand from the 3′ arm, while the miRNA scaffold uses the 5′ arm. However, this order can sometimes be reversed, such as with repetitive sequences. We cloned control shRNAs and miRNA mimics that have no target (shSCRMBL), target mCherry (shMCHR-1), or target CAG repeats with perfect complementarity (shHD1-1). Several shRNAs were then cloned based on the shHD1-1 design, but containing central mismatches. We also cloned small RNAs into partial or full miRNA33 scaffolds,[20] such as miHD-33part and miHD-33full, respectively (Figure 1D).

For the allele-selective mechanism of mismatch-containing small RNAs targeting repeats, mismatches should be placed predictably and consistently. Shifted positioning might compromise target binding by disrupting seed pairing or allowing Ago2 cleavage, which decreases repeat selectivity.[17] To characterize processing precision and relative abundance, vectors were transiently transfected into HEK 293T cells and transfection efficiency quantified by microscopy-based image analysis of GFP and nuclear staining. Small RNAs were then

isolated and sequenced by Illumina (Figures 1D, S1B, and S2A, and Table S2). Variants of shHD1L-1 had different processing precision. For example, loss of duplex pairing (shHD1L-1a) disrupted precision, whereas introduction of miR30 (shHD1L-1b) or miR33 (shHD1L-1c) loops rescued precision (Figure 1D). Small RNAs cloned into partial miR33 scaffolds were not processed correctly, while full miR33 scaffolds (miHD-33full and miHD-33full-mimic) resulted in high 5′ processing precision (Figure 1D). Reduced abundances often correlated with poorer processing.

Selective reduction of a CAG repeat expansion luciferase reporter with vectorized small RNAs

To evaluate the potential for allele selectivity, fragments of exon 1 from wtHTT or mutHTT mRNAs from patient-derived HD fibroblasts with 17 or 69 repeats (HD17/69) were cloned in-frame with firefly luciferase, resulting in 17, 38, and 68 CAG repeat reporter constructs. Reporter vectors were then co-transfected into HEK 293T cells with each small RNA expression vector and luciferase activity normalized to shSCRMBL control (Figures 1E and S3A).

Positive controls shMCHR-1 (Table S1) and shHD1-1 displayed similar decreases in luciferase activity for all repeat sizes. These small RNAs lack central mismatches and elicit target cleavage,[17,19] resulting in indiscriminate reduction. The four miR33 scaffolded small RNAs all provided some degree of selective knockdown of $(CAG)_{38}$ and $(CAG)_{68}$ reporters (Figures 1E and S3A). However, two small RNAs, miHD-33part and miHD-33part-3′k, showed quite poor 5′ processing precision, suggesting that even low levels of properly processed guides may be sufficient for activity and the expression scaffold may be a stronger predictor of repeat selectivity.

To further explore small RNA design iterations, we cloned additional versions of miHD-33full. These were transiently transfected into HEK 293T cells and small RNAs were extracted and sequenced as before (Figures 2A and S2A, and Table S2). A new control shRNA, shHD2.4, was included that targets outside the repeat region with perfect complementarity to cleave both alleles.[26] All miHD-33full variants offered relatively high guide strand processing precision (86%–96%) and moderate to high abundance. We, therefore, chose to directly test these new designs in HD patient-derived fibroblasts.

Allele-selective mutHTT knockdown in patient-derived fibroblasts

We sought to first confirm allele-selective knockdown of mutHTT using mismatch-containing siRNAs[11,17] before testing our vectorized small RNAs. As expected, western blot (WB) analyses revealed non-allele-selective knockdown of HTT protein in patient-derived HD17/69 fibroblasts when using siHD2.4 and siHD3 (Figures S4A and S4B). Conversely, both siHD3-9 and siHD3-9-11, which introduce central mismatches, provided a window of allele-selective knockdown across most concentrations (Figures S4C and S4D). We then transduced HD17/69 fibroblasts with small RNA expression vectors packaged into lentiviral particles and confirmed high infection efficiency by microscopy and flow cytometry (Figures S5A and S5B). HTT protein was then assayed at 7 days after infection by WB quantification (Figures 2B and S3B). shHD2.4 nearly eliminated detectable HTT protein and only one repeat-targeting small RNA, miHD-33full_30lp2, did not provide allele selectivity. Three were further transduced into patient-derived HD15/47 cells, with a smaller $(CAG)_{47}$ mutHTT allele and again conferred significant allele selectivity (Figures 2C and S3C). We then cloned miHD-33full-mimic, a design we sought to carry through all assays, into an AAV vector. When packaged into AAV particles and transduced into HD17/69 cells, miHD-33full-mimic provided significant allele-selective knockdown of mutHTT (Figures S3D and S3E).

CAG repeat-targeted small RNAs induce varying degrees of off-target effects

Exogenous small RNAs have the potential to perturb endogenous miRNA profiles or induce off-target effects on gene expression. Small RNA sequencing (RNA-seq) revealed relatively modest changes to the abundance of the top five miRNAs in HEK 293T cells for miHD-33full-mimic, miHD-33full_nb, and miHD-33full_UAC, small RNAs that were allele selective in HD17/69 and HD15/47 cells (Figure S2B), suggesting a low likelihood of altering small RNA regulation. The expression of other CAG repeat-containing genes[27] may be impacted by CAG-targeting small RNAs. For example, *TATA binding protein* (*TBP*) contains a relatively long tract of $(CAG)_{19}$. However, when TBP protein levels were evaluated by WB after lentiviral transduction of HD17/69 cells, expression was not significantly changed (Figures S6A and S6B).

To assess potential transcriptome-wide off-target effects, we performed nanopore long-read mRNA-seq. HD17/69 cells were transduced with lentiviral particles expressing mock (no small RNA), miMCHRY-33full-mimic, shHD2.4, miHD-33full-mimic, miHD-33full_nb, or miHD-33full_UAC. After mRNA-seq from these treatments, global differential gene expression (DGE) was normalized to miMCHRY-33full-mimic. Surprisingly, miHD-33full-mimic exhibited more than an order of magnitude fewer differentially expressed genes compared with shHD2.4, miHD-33full_nb, and miHD-33full_UAC (Figures 3A–3D, S6C, and S6D). Lentiviral transduction was efficient (Figure S7) and HTT mRNA was reduced to similar levels for all three CAG-targeted small RNAs (Figure S8), suggesting that small RNA expression does not account for the large discrepancy in off-target effects. Most differentially expressed genes for CAG-targeted small RNAs are unlikely to be attributable to loss of HTT,[9] since normalization to shHD2.4 still presented substantial DGE (Figure S9), supporting unique off-targets for miHD-33full_nb and miHD-33full_UAC (Figure S10A). The mRNA levels of several known CAG repeat-containing genes[27] were largely unaffected by CAG-targeting small RNAs, supporting a role for flanking sequence context[14,18] (Figures 3E–3H). However, some were significantly decreased, including CIZ1, MED15, and AF9/MLLT3 (Figure S8). CAG-repeat-targeting small RNAs also shared many of the top differentially expressed genes (Figure S10B), indicating a shared off-target mechanism. One source of off-target effects may be passenger strand targeting, especially for repetitive small RNAs that could neutralize

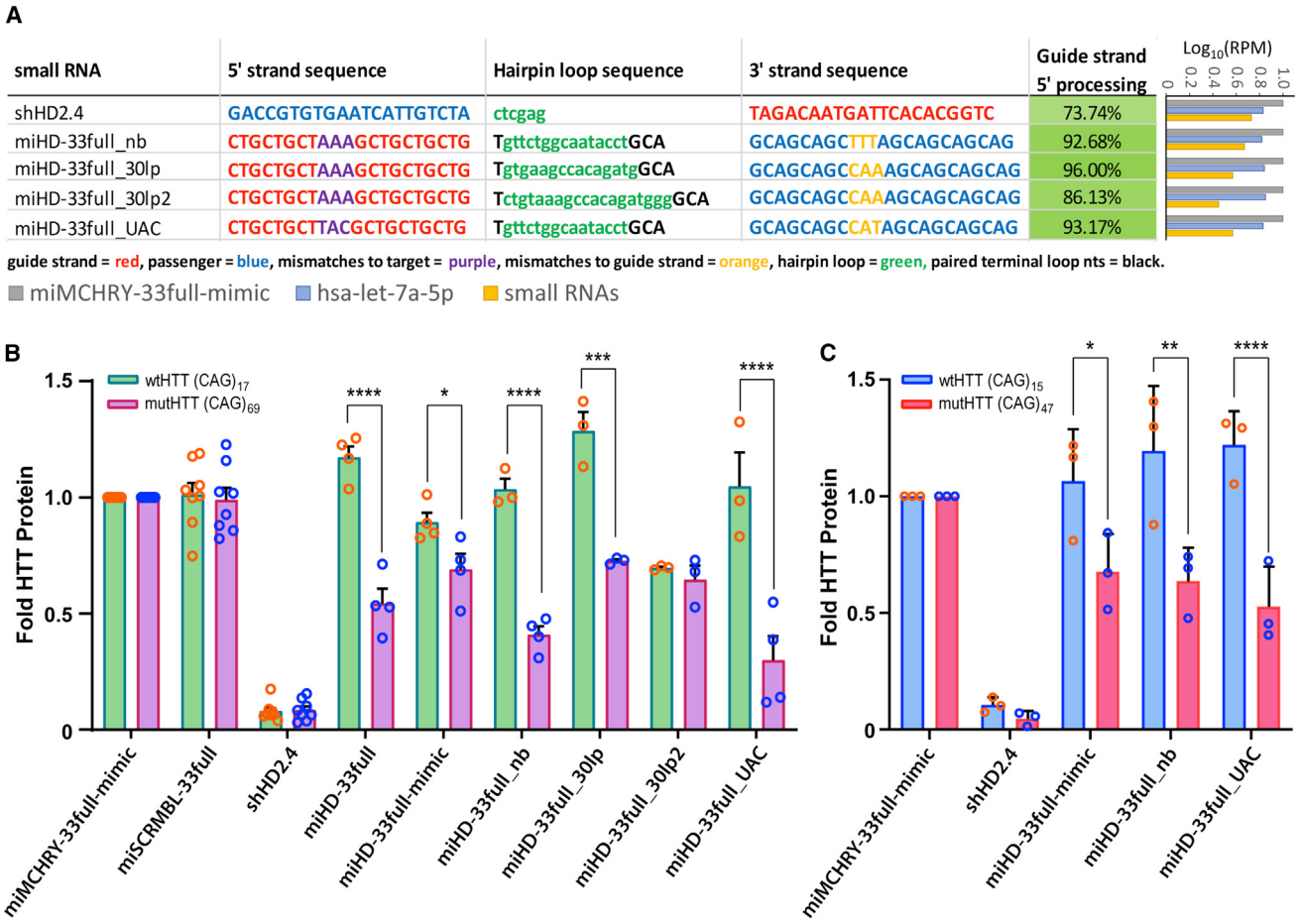


Figure 2. Sequencing of second-generation small RNAs expressed in HEK 293T cells and their allele selectivity in patient-derived HD fibroblasts

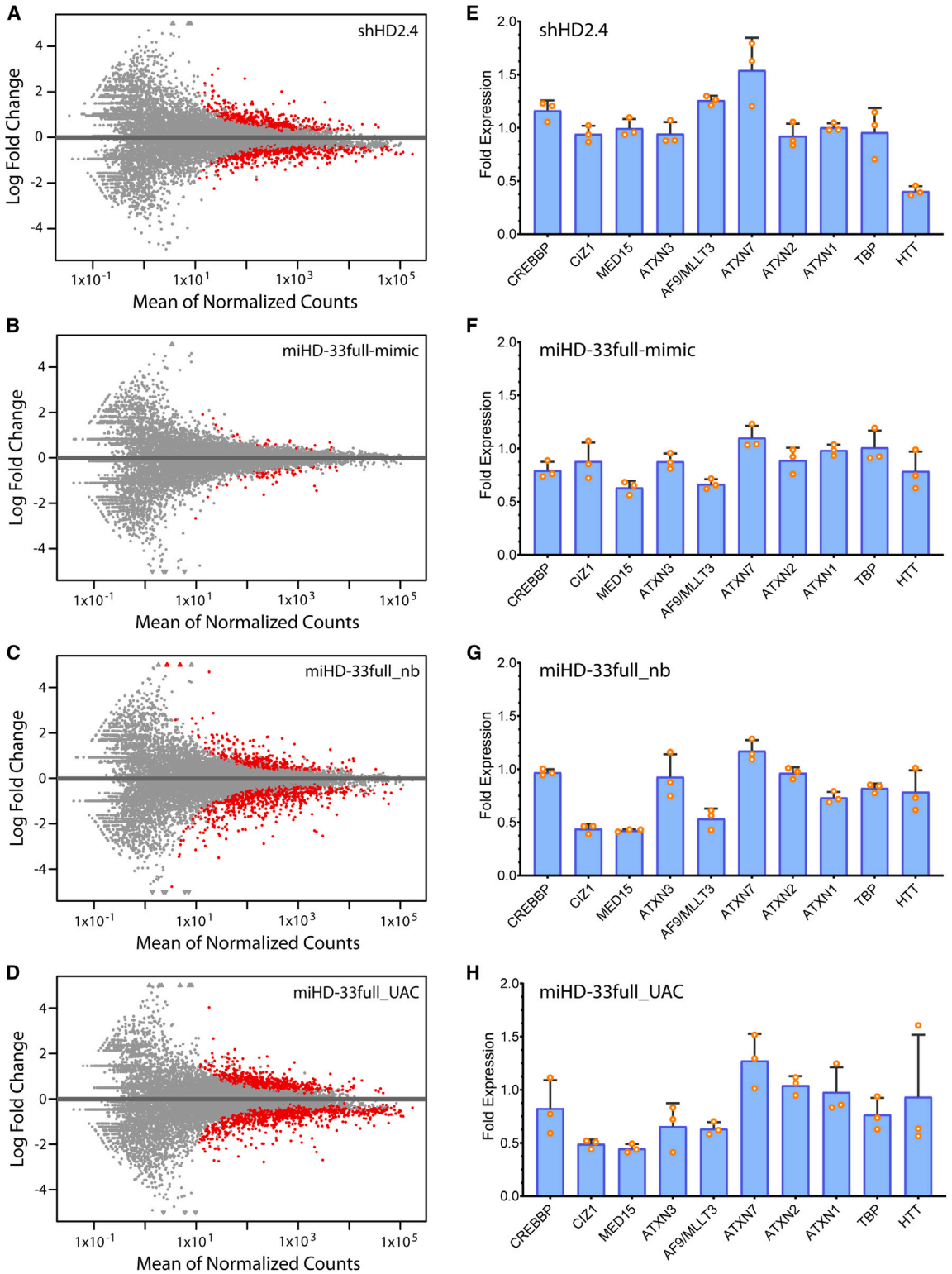
(A) DNA-level sequence of the 5′ arm, hairpin loop, and 3′ arm of small RNA expression constructs. The 5′ guide strand processing precision and relative abundance (plot to the right) are normalized to a control miMCHRY-33full-mimic small RNA construct (expressing non-targeting mCherry small RNA) and are reported based on small RNA-seq results after transient transfection of expression constructs in HEK 293T cells. (B) Quantification of WBs for HTT protein 7 days after lentiviral transduction of small RNA expression constructs in patient-derived HD17/69 fibroblasts. Error bars are standard error of the mean (S.E.M.). NS = not significant, *p ≤ 0.05, ***p < 0.001, ****p < 0.0001. (C) Quantification of WBs for HTT protein 7 days after lentiviral transduction of small RNA expression constructs in patient-derived HD15/47 fibroblasts. Error bars are SEM. NS, not significant. *p ≤ 0.05, **p < 0.01, ****p < 0.0001.

Ago strand loading bias.[11,15,17,18] miHD-33full-mimic and miHD-33full_nb only differ by their passenger strand sequences and shHD2.4, miHD-33full_nb, and miHD-33full_UAC, with the greatest off-target effects, are processed into a much broader diversity of passenger strand sizes and sequences compared with miHD-33full-mimic (Table S2).

DISCUSSION

The eventual success of therapeutic small nucleic acids for treatment of HD will likely rely on many factors, including patient staging and stratification, on- and off-target engagement, and possibly allele selectivity for mutHTT.[7,8,28] To help better evaluate allele-selective small RNAs as a potential gene therapy, we genetically encoded and characterized CAG repeat-targeted small RNAs with central mismatches. Several designs, especially based on the full miR33 scaffold, achieved favorable 5′ processing of the guide strand and selectively reduced mutHTT in patient-derived cells. Although accurate placement of mismatches in the guide strand is expected to be critical for allele selectivity,[10,13] the favorable processing but poor selectivity of some designs, such as shRNA scaffolds and miHD-33full_30lp2, suggest that other factors may be involved. Similarly, the processing precision and sequence of passenger strands may be an important contributor to off-target effects.[29] Clearer rules will likely emerge by testing a broader range of variables, including more loop sequences, central mismatch sequences, or miRNA scaffolds.[22]

This study highlights the value of adopting sequencing methods to balance allele selectivity, potency, and safety during the design and selection of CAG repeat-targeted small RNAs for potential therapeutic applications. For example, in this study miHD-33full_nb and



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miHD-33full_UAC seemed to be better candidates due to strong allele selectivity. However, mRNA-seq indicated that miHD-33full-mimic is likely to be a better option for therapeutic development due to dramatically lower off-target effects. In addition to robust activity and allele selectivity, designs should strive to achieve consistent and precise processing precision of both guide and passenger strands as well as take into account endogenous miRNA and global DGE profiles.

MATERIALS AND METHODS

Materials and methods are provided in the online Supplemental information.

DATA AND CODE AVAILABILITY

Sequencing data and accessory files are freely available through the National Institutes of Health (NIH) Gene Expression Omnibus (GEO), GEO: GSE242506.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2024.102206.

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AUTHOR CONTRIBUTIONS

M.A.P. prepared samples for small RNA sequencing sample preparation, transfected and infected cells, developed and performed HTT WBs, performed microscopy and flow cytometry, analyzed and interpreted data, prepared figures, and assisted with experimental design, writing, and preparation of the manuscript. A.A.W. cloned small RNA expression constructs, helped to prepare small RNA samples for sequencing, analyzed small RNA sequencing results, prepared samples for mRNA-seq, performed mRNA-seq, analyzed mRNA-seq, prepared figures, and assisted with experimental design and writing of the manuscript. R.C. developed small RNA expression cloning protocols, cloned small RNA expression constructs, and cloned HTT exon 1 fragments for preparation of luciferase expression reporter constructs. A.A.P. developed the initial small RNA sequencing analysis workflow, analyzed small RNA sequencing results, and supported mRNA-seq with protocols and analysis pipelines. F.E. transfected cells, prepared samples for small RNA sequencing, and developed and performed HTT and off-target WB experiments. K.N.O. transfected cells and prepared small RNA samples for sequencing. P.J.J. designed experiments, assisted in small RNA expression construct design and cloning, cloned luciferase reporter constructs, performed luciferase assays, developed and performed HTT WBs, performed microscopy, packaged vectors into lentivirus particles, prepared AAV reagents and performed AAV experiments, performed transfections and infections, prepared figures, and helped to write the manuscript and supervise the project. K.T.G. assisted with HTT WB development and experiments, designed small RNA expression scaffolds and constructs, assisted in small RNA sample preparation for sequencing, prepared figures, wrote the manuscript, and helped conceive of and supervise the project.

DECLARATION OF INTERESTS

K.T.G. is a scientific co-founder of Iris Medicine. K.T.G and P.J.J. have a patent related to this work.

REFERENCES

1. Depienne, C., and Mandel, J.L. (2021). 30 years of repeat expansion disorders: What have we learned and what are the remaining challenges? Am. J. Hum. Genet. *108*, 764–785.
2. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. Cell *72*, 971–983.
3. Groc, L., and Choquet, D. (2020). Linking glutamate receptor movements and synapse function. Science *368*, eaay4631.
4. Jurcau, A. (2022). Molecular Pathophysiological Mechanisms in Huntington's Disease. Biomedicines *10*, 1432.
5. Migazzi, A., Scaramuzzino, C., Anderson, E.N., Tripathy, D., Hernández, I.H., Grant, R.A., Roccuzzo, M., Tosatto, L., Virlogeux, A., Zuccato, C., et al. (2021). Huntingtin-mediated axonal transport requires arginine methylation by PRMT6. Cell Rep. *35*, 108980.
6. Thakur, A.K., Jayaraman, M., Mishra, R., Thakur, M., Chellgren, V.M., Byeon, I.J.L., Anjum, D.H., Kodali, R., Creamer, T.P., Conway, J.F., et al. (2009). Polyglutamine disruption of the huntingtin exon 1 N terminus triggers a complex aggregation mechanism. Nat. Struct. Mol. Biol. *16*, 380–389.
7. Leavitt, B.R., Kordasiewicz, H.B., and Schobel, S.A. (2020). Huntingtin-Lowering Therapies for Huntington Disease: A Review of the Evidence of Potential Benefits and Risks. JAMA Neurol. *77*, 764–772.
8. Kingwell, K. (2021). Double setback for ASO trials in Huntington disease. Nat. Rev. Drug Discov. *20*, 412–413.
9. Bensalel, J., Xu, H., Lu, M.L., Capobianco, E., and Wei, J. (2021). RNA-seq analysis reveals significant transcriptome changes in huntingtin-null human neuroblastoma cells. BMC Med. Genomics *14*, 176.
10. Gagnon, K.T., Pendergraff, H.M., Deleavey, G.F., Swayze, E.E., Potier, P., Randolph, J., Roesch, E.B., Chattopadhyaya, J., Damha, M.J., Bennett, C.F., et al. (2010). Allele-selective inhibition of mutant huntingtin expression with antisense oligonucleotides targeting the expanded CAG repeat. Biochemistry *49*, 10166–10178.
11. Yu, D., Pendergraff, H., Liu, J., Kordasiewicz, H.B., Cleveland, D.W., Swayze, E.E., Lima, W.F., Crooke, S.T., Prakash, T.P., and Corey, D.R. (2012). Single-stranded

Figure 3. DGE analysis and knockdown of known CAG repeat-containing genes after lentiviral transduction of patient-derived HD fibroblasts

(A–D) Volcano plots illustrating differentially expressed genes (red dots) from mRNA-seq in patient-derived HD17/69 fibroblasts 7 days after lentiviral transduction. Log fold change is normalized to miMCHRY-33full-mimic baseline expression values with a p value cutoff of <0.05. (E–H) Relative mRNA levels for known CAG repeat-containing genes taken from global mRNA-seq data used in (A–D). Expression values are normalized to miMCHRY-33full-mimic values for each gene. Genes are shown from left to right based on fewest to greatest number of tandem CAG repeats. Error bars are SEM.

RNAs use RNAi to potently and allele-selectively inhibit mutant huntingtin expression. Cell *150*, 895–908.

12. Hu, J., Matsui, M., and Corey, D.R. (2009). Allele-selective inhibition of mutant huntingtin by peptide nucleic acid-peptide conjugates, locked nucleic acid, and small interfering RNA. Ann. N. Y. Acad. Sci. *1175*, 24–31.

13. Miniarikova, J., Zanella, I., Huseinovic, A., van der Zon, T., Hanemaaijer, E., Martier, R., Koornneef, A., Southwell, A.L., Hayden, M.R., van Deventer, S.J., et al. (2016). Design, Characterization, and Lead Selection of Therapeutic miRNAs Targeting Huntingtin for Development of Gene Therapy for Huntington's Disease. Mol. Ther. Nucleic Acids *5*, e297.

14. Ciesiolka, A., Stroynowska-Czerwinska, A., Joachimiak, P., Ciolak, A., Kozlowska, E., Michalak, M., Dabrowska, M., Olejniczak, M., Raczynska, K.D., Zielinska, D., et al. (2021). Artificial miRNAs targeting CAG repeat expansion in ORFs cause rapid deadenylation and translation inhibition of mutant transcripts. Cell. Mol. Life Sci. *78*, 1577–1596.

15. Fiszer, A., Olejniczak, M., Galka-Marciniak, P., Mykowska, A., and Krzyzosiak, W.J. (2013). Self-duplexing CUG repeats selectively inhibit mutant huntingtin expression. Nucleic Acids Res. *41*, 10426–10437.

16. Monteys, A.M., Wilson, M.J., Boudreau, R.L., Spengler, R.M., and Davidson, B.L. (2015). Artificial miRNAs Targeting Mutant Huntingtin Show Preferential Silencing In Vitro and In Vivo. Mol. Ther. Nucleic Acids *4*, e234.

17. Hu, J., Liu, J., and Corey, D.R. (2010). Allele-selective inhibition of huntingtin expression by switching to an miRNA-like RNAi mechanism. Chem. Biol. *17*, 1183–1188.

18. Kotowska-Zimmer, A., Ostrovska, Y., and Olejniczak, M. (2020). Universal RNAi Triggers for the Specific Inhibition of Mutant Huntingtin, Atrophin-1, Ataxin-3, and Ataxin-7 Expression. Mol. Ther. Nucleic Acids *19*, 562–571.

19. Hu, J., Matsui, M., Gagnon, K.T., Schwartz, J.C., Gabillet, S., Arar, K., Wu, J., Bezprozvanny, I., and Corey, D.R. (2009). Allele-specific silencing of mutant huntingtin and ataxin-3 genes by targeting expanded CAG repeats in mRNAs. Nat. Biotechnol. *27*, 478–484.

20. Xie, J., Tai, P.W.L., Brown, A., Gong, S., Zhu, S., Wang, Y., Li, C., Colpan, C., Su, Q., He, R., et al. (2020). Effective and Accurate Gene Silencing by a Recombinant AAV-Compatible MicroRNA Scaffold. Mol. Ther. *28*, 422–430.

21. Fang, W., and Bartel, D.P. (2015). The Menu of Features that Define Primary MicroRNAs and Enable De Novo Design of MicroRNA Genes. Mol. Cell *60*, 131–145.

22. Gu, S., Jin, L., Zhang, Y., Huang, Y., Zhang, F., Valdmanis, P.N., and Kay, M.A. (2012). The loop position of shRNAs and pre-miRNAs is critical for the accuracy of dicer processing in vivo. Cell *151*, 900–911.

23. Kotowska-Zimmer, A., Przybyl, L., Pewinska, M., Suszynska-Zajczyk, J., Wronka, D., Figiel, M., and Olejniczak, M. (2022). A CAG repeat-targeting artificial miRNA lowers the mutant huntingtin level in the YAC128 model of Huntington's disease. Mol. Ther. Nucleic Acids *28*, 702–715.

24. Moffat, J., Grueneberg, D.A., Yang, X., Kim, S.Y., Kloepfer, A.M., Hinkle, G., Piqani, B., Eisenhaure, T.M., Luo, B., Grenier, J.K., et al. (2006). A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell *124*, 1283–1298.

25. Chilamkurthy, R., White, A.A., Pater, A.A., Jensik, P.J., and Gagnon, K.T. (2022). Efficient Cloning and Sequence Validation of Repetitive and High GC-Content Short Hairpin RNAs. Hum. Gene Ther. *33*, 829–839.

26. Boudreau, R.L., McBride, J.L., Martins, I., Shen, S., Xing, Y., Carter, B.J., and Davidson, B.L. (2009). Nonallele-specific silencing of mutant and wild-type huntingtin demonstrates therapeutic efficacy in Huntington's disease mice. Mol. Ther. *17*, 1053–1063.

27. Butland, S.L., Devon, R.S., Huang, Y., Mead, C.L., Meynert, A.M., Neal, S.J., Lee, S.S., Wilkinson, A., Yang, G.S., Yuen, M.M.S., et al. (2007). CAG-encoded polyglutamine length polymorphism in the human genome. BMC Genom. *8*, 126.

28. Thomson, S.B., Stam, A., Brouwers, C., Fodale, V., Bresciani, A., Vermeulen, M., Mostafavi, S., Petkau, T.L., Hill, A., Yung, A., et al. (2023). AAV5-miHTT-mediated huntingtin lowering improves brain health in a Huntington's disease mouse model. Brain *146*, 2298–2315.

29. Sheng, P., Flood, K.A., and Xie, M. (2020). Short Hairpin RNAs for Strand-Specific Small Interfering RNA Production. Front. Bioeng. Biotechnol. *8*, 940.