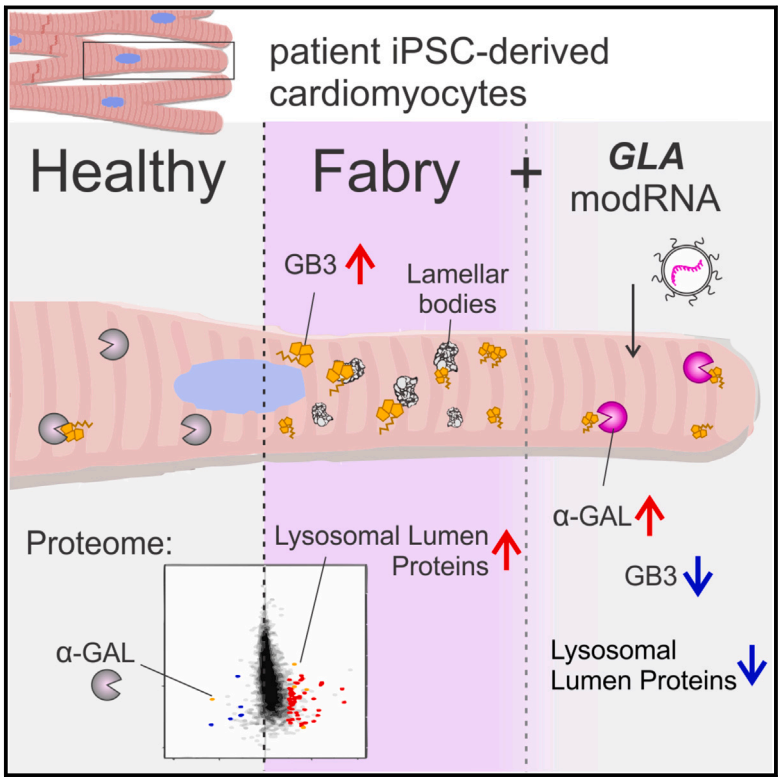
GLA-modified RNA treatment lowers GB3 levels in iPSC-derived cardiomyocytes from Fabry-affected individuals

Graphical abstract



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Nucleoside-modified messenger RNA (modRNA) has shown great promise as an enzyme replacement tool. Here, we show that GLA modRNA almost completely abolishes the accumulation of globotriaosylceramides observed in cardiomyocytes derived from individuals with Fabry disease. Moreover, changes in the proteome between Fabry and healthy cardiomyocytes were rescued upon modRNA treatment.

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REPORT

GLA-modified RNA treatment lowers GB3 levels in iPSC-derived cardiomyocytes from Fabry-affected individuals

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Summary

Recent studies in non-human model systems have shown therapeutic potential of nucleoside-modified messenger RNA (modRNA) treatments for lysosomal storage diseases. Here, we assessed the efficacy of a modRNA treatment to restore the expression of the galactosidase alpha (*GLA*), which codes for α-Galactosidase A (α-GAL) enzyme, in a human cardiac model generated from induced pluripotent stem cells (iPSCs) derived from two individuals with Fabry disease. Consistent with the clinical phenotype, cardiomyocytes from iPSCs derived from Fabry-affected individuals showed accumulation of the glycosphingolipid Globotriaosylceramide (GB3), which is an α-galactosidase substrate. Furthermore, the Fabry cardiomyocytes displayed significant upregulation of lysosomal-associated proteins. Upon *GLA* modRNA treatment, a subset of lysosomal proteins were partially restored to wild-type levels, implying the rescue of the molecular phenotype associated with the Fabry genotype. Importantly, a significant reduction of GB3 levels was observed in *GLA* modRNA-treated cardiomyocytes, demonstrating that α-GAL enzymatic activity was restored. Together, our results validate the utility of iPSC-derived cardiomyocytes from affected individuals as a model to study disease processes in Fabry disease and the therapeutic potential of *GLA* modRNA treatment to reduce GB3 accumulation in the heart.

Fabry disease (MIM: 301500) is a rare X-linked lysosomal storage disease that can be caused by a wide range of pathogenic mutations in galactosidase alpha (*GLA* [MIM: 300644]). In healthy cells, *GLA* encodes the α-Galactosidase A (α-GAL) enzyme that catalyzes the cleavage of the terminal galactose from the glycosphingolipid Globotriaosylceramide (GB3). Individuals with Fabry disease display reduced α-GAL enzyme activity leading to an accumulation of GB3 in lysosomes.[1,2] GB3 accumulates in lysosomes within all cell types in Fabry-affected individuals resulting in a wide range of clinical symptoms. While chronic kidney disease is a leading cause of death, many individuals with Fabry disease die as a result of heart failure due to diastolic dysfunction.[3,4] At present, enzyme-replacement therapy is considered the best standard of care for persons with Fabry disease. However, despite alleviation of clinical symptoms, some individuals still suffer from renal or cardiac manifestations.[5] Moreover, enzyme-replacement therapy has been reported to induce an immune response in some individuals, which can result in reduced enzymatic activity and progressing accumulation of GB3[6,7] or in infusion-associated reactions.[8] In addition, the high cost of enzyme-replacement therapy limits the widespread use of this approach. Small molecule chaperone therapy, on the other hand, is a viable option only for individuals with a missense mutation that results in a misfolded α-GAL protein, which covers only 35%–50% of all persons with Fabry disease.[9] Therefore, alternative therapeutic approaches would benefit this population. Here, we use pluripotent stem cell technology to determine whether nucleoside-modified messenger RNA (modRNA) encoding α-GAL may alleviate the cellular phenotype of Fabry disease-related cardiomyopathy.

modRNA has been used clinically to produce therapeutic proteins, most notably as vaccines for COVID-19.[10] Moreover, modRNA has been proposed as a gene replacement therapy for inherited disorders including Fabry disease.[11] Although animal models have demonstrated the potential

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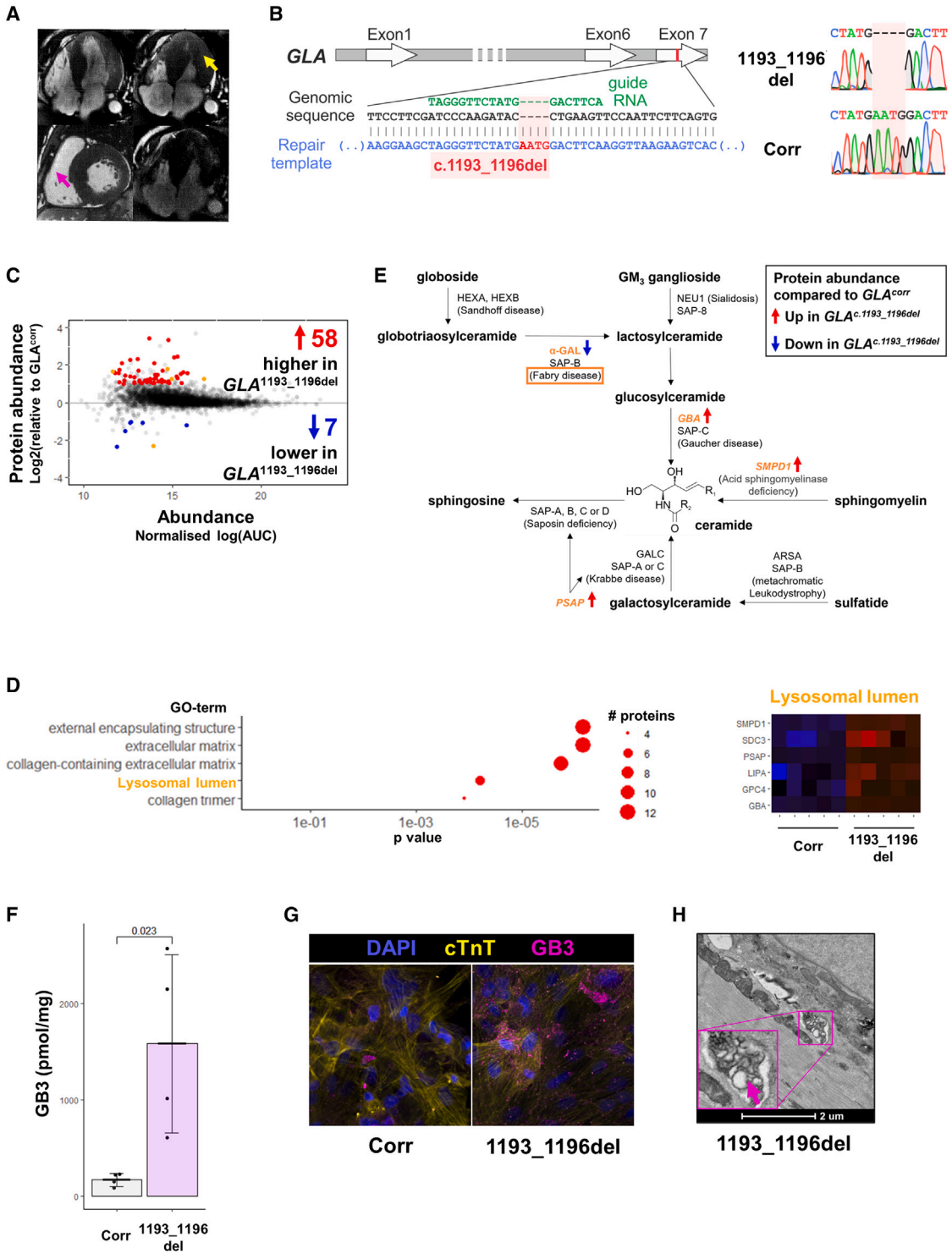

Figure 1. Recapitulation of Fabry disease in induced pluripotent stem cell-derived cardiomyocytes

(A) Cardiac MRI showing marked symmetric enlargement of the left ventricle (top, yellow arrow) and myocardial inflammation and myocardial fibrosis (bottom, pink arrow).

(B) Schematic showing the method for correcting the c.1193_1196del mutation in *GLA* in induced pluripotent stem cells derived from an individual with Fabry disease.

(C) MA-plot indicating the proteins with differential levels between $GLA^{c.1193\_1196del}$ Fabry and GLA^{corr} isogenic control cardiomyocytes (log2-fold-change > 1, adjusted p value < 0.05).

(D) GO-term analysis reveals a significant enrichment of proteins involved in the lysosomal pathway within the total number of proteins higher in the $GLA^{c.1193\_1196del}$ Fabry cardiomyocytes when compared to GLA^{corr} isogenic control (left). The normalized abundance of these proteins is shown in a heatmap (right).

(legend continued on next page)

of modRNA to restore α-GAL activity in Fabry disease following systemic delivery,[12] this approach has not been tested in a human model of Fabry disease and the effects on cardiac muscle are not clear. To assess the potential of *GLA* modRNA as a therapeutic agent in Fabry disease, we developed an induced pluripotent stem cell (iPSC) model of Fabry cardiomyopathy. The person, a 57-year-old male, presented at age 49 with impaired renal function, the presence of focal glomerulosclerosis with interstitial fibrosis, and glomerular lipid accumulation leading to the diagnosis of Fabry disease. Diagnosis was confirmed by very low levels of α-GAL activity (0.2 nmol/h/mL, compared to 4.2–17.3 nmol/h/L in unaffected individuals[13]) and a deletion mutation in *GLA* ($GLA^{1193\_1196del}$; GenBank: NC_000023.11, NM_000169.2: c.1193_1196del [p.Glu398Glyfs*5]), resulting in frameshift and a premature stop codon. Echocardiography revealed severe left ventricular hypertrophy and diastolic dysfunction (Figure 1A).

To study the pathogenesis of Fabry disease using pluripotent stem cell models from an individual with the $GLA^{c.1193\_1196del}$ variant, we generated (1) an affected-individual-specific iPSC line and (2) an isogenic control cell line in which we repaired the c.1193_1196del lesion. Informed consent was obtained under the Royal Melbourne Hospital Human Research Ethics Committee approval 66294/MH-2020. The $GLA^{c.1193\_1196del}$ iPSC line was generated via messenger RNA-based overexpression of the Yamanaka reprogramming factors, and the isogenic control cell line, GLA^{corr}, was subsequently generated via CRISPR-Cas9-mediated homology directed repair (Figure 1B). Both the $GLA^{c.1193\_1196del}$ and the GLA^{corr} cell lines were karyotypically normal, maintained expression of pluripotency markers after genetic modification, and had comparable cardiac differentiation capacity (Figures S1A–S1C and Videos S1 and S2). Therefore, we have generated isogenic cell lines varying only in c.1193_1196 deletion of *GLA*.

To assess whether cardiomyocytes (CMs) differentiated from $GLA^{c.1193\_1196del}$ iPSCs recapitulate the cellular phenotype observed in tissue from affected individuals, we performed label-free proteomics to identify biochemical differences between Fabry and isogenic control iPSC-derived CMs. A total of 5,171 proteins were identified across all conditions. We identified 65 proteins with differential abundance (log2 fold change > 1; adjusted p value of 0.05) which was <2% of the total number of proteins detected. The majority (58) were upregulated in $GLA^{c.1193\_1196del}$ CMs (Figure 1C; Table S1). As expected, α-GAL was one of the top downregulated proteins in the Fabry line when compared to its isogenic control (GLA^{corr}). Gene ontology (GO)-term analysis on the proteins with higher levels in the $GLA^{c.1193\_1196del}$ CMs revealed a significant enrichment for lysosomal proteins (6/65 proteins, adjusted p value $< 6.27e{-}05$), including GBA (MIM: 606463) which was previously shown to be upregulated in cardiomyocytes from individuals with Fabry disease,[14] suggesting that these iPSC-derived CMs recapitulate the Fabry disease phenotype (Figures 1D and 1E). To confirm, we next quantified GB3 accumulation in the Fabry line and its isogenic control. Lipidomic profiling demonstrated that the GLA^{corr} isogenic control line did not display the same elevated GB3 levels observed in $GLA^{c.1193\_1196del}$ CMs (Figure 1F). An increase in peri-nuclear accumulation of GB3-positive vesicles in $GLA^{c.1193\_1196del}$ cardiomyocytes was confirmed by confocal microscopy (Figure 1G). In line with the mass spectrometry data, GB3-positive vesicles were rarely observed in isogenic control lines. Further, transmission electron microscopy revealed the presence of lamellar bodies in cardiomyocytes from $GLA^{c.1193\_1196del}$ that were not observed in GLA^{corr} isogenic control (Figures 1H and S1D). Notably, despite the clear accumulation of GB3, we did not observe a difference in contractility between Fabry iPSC cardiac organoids and their isogenic controls (data not shown), which is consistent with the mid-life cardiomyopathy onset observed in this person. Together, these results show that the established cellular phenotypes of Fabry disease, including accumulation of GB3, are recapitulated in $GLA^{c.1193\_1196del}$ iPSC-derived CMs, validating their utility to study candidate therapeutic interventions.

modRNA has emerged as a potential therapeutic approach to substitute for enzyme-replacement therapy with several studies showing that the heart is amenable to modRNA treatment.[11] A recent study in mice and non-human primates demonstrated that intravenous administration of *GLA* modRNA results in the production of therapeutically active enzyme and a reduction of GB3 levels in tissues including the heart.[12] Importantly, the treatment did not result in the production of neutralizing antibodies, suggesting that the approach may confer benefits over enzyme-replacement therapy. We aimed to test whether *GLA* modRNA therapy can rescue the molecular phenotype in our human iPSC-based model of Fabry disease (Figure 2). $GLA^{c.1193\_1196del}$ CMs were treated with modRNA encoding for α-GAL, or modRNA encoding the green fluorescent protein (GFP) as a negative control (Figure 2A). modRNA was formulated in lipid nanoparticles as a delivery vector. GFP was detected in both cardiomyocytes and non-myocytes, indicating efficient lipid

(E) Schematic of the sphingolipid catabolic pathway highlighting the differential proteins ($GLA^{c.1193\_1196del}$ Fabry cardiomyocytes versus GLA^{corr} isogenic control cardiomyocytes).
(F) Mass spectrometry analysis showing an increase in the summed level of GB3 in $GLA^{c.1193\_1196del}$ Fabry cardiomyocytes when compared to GLA^{corr} isogenic control. Statistical significance was calculated using the t test. Error bars represent the standard deviation.
(G) GB3-positive vesicles in cTnT-positive Fabry cardiomyocytes.
(H) Transmission electron microscopy image showing lamellar bodies in cardiomyocytes.

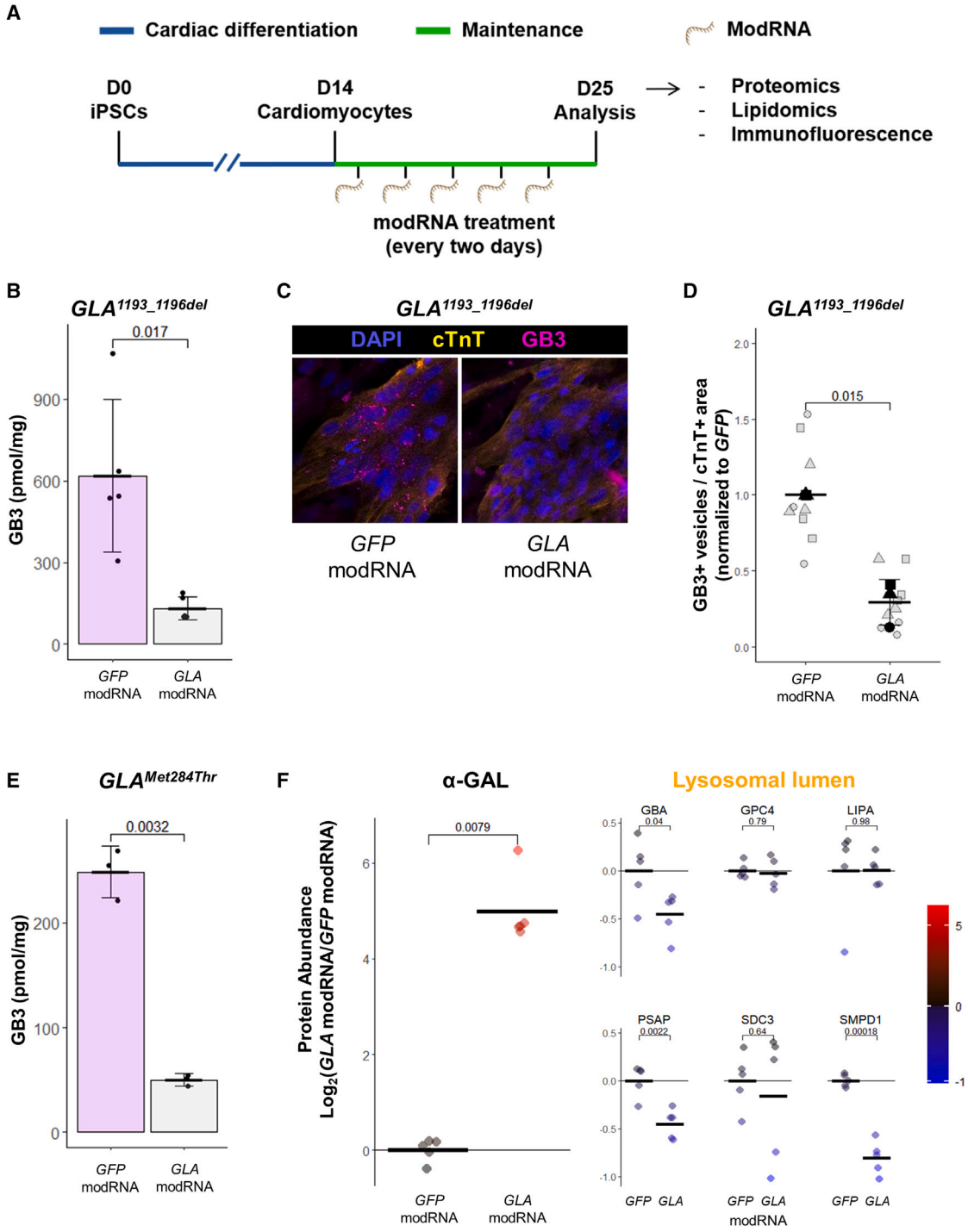

Figure 2. Treatment with *GLA*-modified RNA rescues Fabry's phenotype in human cardiomyocytes *in vitro*

(A) Overview of the approach taken to test the efficacy of *GLA* modRNA to rescue the Fabry disease phenotype.

(B) GB3 levels as observed by lipidomics analysis.

(C) Immunofluorescence imaging reveals a reduction in GB3 in *GLA* modRNA-treated cardiomyocytes.

(D) Image-based quantification of GB3 shows a reduction in the number of GB3-positive vesicles in GLA modRNA-treated versus GFP modRNA-treated Fabry cardiomyocytes. Data of three independent experiments, as indicated by shape, each consisting of three technical replicates.

(E) Mass spectrometry confirming a reduction in total GB3 upon *GLA* modRNA treatment in iPSC-derived cardiomyocytes from a Fabry individual with a p.Met284Thr mutation.

(F) Proteomics data indicating an increase in the level of α-GAL and a partial reduction in lysosomal proteins upon *GLA* modRNA treatment.

In (B), (D), (E), and (F), significance was tested using a t test. Error bars represent standard deviation.

nanoparticle-mediated targeting (Figure S2A). Furthermore, immunofluorescence labeling showed that α-GAL was successfully increased in the *GLA* modRNA-treated samples, demonstrating the efficiency of this method (Figure S2A).

We next assessed whether the *GLA* modRNA could rescue the GB3 accumulation observed in $GLA^{c.1193\_1196del}$ CMs. Mass spectrometry analysis showed a significant reduction in GB3 upon treatment with the *GLA* modRNA (Figure 2B). Immunofluorescence on GB3 confirmed these data and showed a significant decrease in the number of GB3-positive vesicles in *GLA* modRNA-treated cardiomyocytes specifically (Figures 2C and 2D). In addition, a reduction in GB3 upon *GLA* modRNA treatment in cardiomyocytes generated from iPSCs derived from another individual ($GLA^{Met284Thr}$ mutation; GenBank: NC_000023.11 and NM_000169.2: c.851T>C [p.Met284Thr]; Figures S2B and S2C, Videos S3 and S4) with severe classical form of Fabry disease (Figure 2E) was shown, confirming the efficacy of the modRNA treatment.

Finally, to assess whether the *GLA* modRNA treatment restores the biochemical differences observed between $GLA^{c.1193\_1196del}$ Fabry and isogenic control CMs, we performed label-free proteomics on CMs from an affected individual transfected with control (*GFP*) or *GLA* modRNA. As expected, α-GAL was the top up-regulated protein (log2 fold change = 4.98, Figure 2F). Focusing on the six lysosomal lumen-associated proteins found to be upregulated in $GLA^{c.1193\_1196del}$ Fabry CMs (Figure 1D, right), three were significantly downregulated upon modRNA treatment (GBA, PSAP [MIM: 611721], and SMPD1 [MIM: 607608], p value < 0.05) (Figure 2F). Interestingly, each of these proteins are involved in the sphingolipid catabolic pathway (Figure 1E), whereas others that were not affected by the treatment, e.g., GPC4 (MIM: 300168) and SDC3 (MIM: 186357), are not. The fact that these proteins are not downregulated upon modRNA treatment might reflect the inability of modRNA-based enzyme-replacement therapies to fully rescue the pathophysiology of Fabry disease. Indeed, GB3 concentrations in *GLA* modRNA-treated Fabry CMs were not reduced to wild-type levels (Figure S2D), which may account for the partial proteomic correction observed (Figure 2F). These results could reflect a common phenomenon where genes affected in disease do not normalize despite correction of the primary defect, as similar findings have been reported for other single-gene lysosomal storage disorders.[15] Alternatively, lack of normalization of gene expression changes in Fabry disease could be driven by epigenetic modifications.[16] Earlier interventions and/or prolonged treatment may be required to ameliorate chromatin modifications in Fabry disease to fully restore transcriptional and proteomic changes in the heart.

As the current gold-standard enzyme-replacement therapy for Fabry disease has certain disadvantages, such as the potential production of neutralizing antibodies and the high costs, significant efforts have been made to develop alternatives, of which several gene-therapy approaches have shown promising results.[17,18] The use of modRNA, which does not integrate in the genome, has recently been shown to efficiently restore α-GAL activity and reduce GB3 accumulation in monkeys and a Fabry mouse model.[12] The findings presented in this report indicate that *GLA* modRNA is efficiently translated into a functional protein in cardiomyocytes derived from iPSCs generated from individuals with Fabry disease. Based on lipidomics and proteomics assays, we demonstrate that modRNA-based *GLA* replacement therapy can rescue the aberrant lysosomal lipid metabolism and accumulation of GB3 in Fabry cardiomyocytes with two different *GLA* variants. While precise targeting of modRNA to specific organs remains challenging,[19] lipid nanoparticle modRNA delivery systems incorporating cell-lineage-specific antibodies are an emerging technology that holds promise for precise delivery of modRNA into organs including the heart and kidney.[20] Furthermore, existing catheter-based delivery could be deployed to improve modRNA treatment of the heart and kidney.[21] In the context of Fabry disease, it is important to note that *GLA* modRNA administered systemically was able to restore α-GAL enzyme activity in both mouse and non-human primate pre-clinical animal models.[12] Importantly, a single dose resulted in a prolonged (>12 weeks) reduction of GB3 levels in the Fabry mouse model, highlighting the long duration of modRNA action.[12] Together, these results support further clinical development of *GLA* modRNA as a treatment modality for cardiac-related pathology in Fabry disease.

Data and code availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD038361.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2023.07.013.

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Author contributions

M.tH., K.M.N., E.R.P., and D.A.E. conceptualized the project. M.tH., B.P., N.Q.L., E.L.Q., C.V., R.J.M., J.E.H., D.A.B., A.W.,

J.T.S., M.F., S.R., and K.I.W. designed and performed experiments. K.M.N. performed analysis of clinical data, patient recruitment, consenting, and biobanking. B.P. performed and analyzed proteomics, M.F. performed and analyzed lipidomics. P.F. and P.G.V.M. contributed to experimental design and provided modified RNAs. K.M.N., E.R.P., and D.A.E. obtained funding. M.tH., E.R.P., and D.A.E. wrote the manuscript. All authors reviewed, edited, and approved the manuscript.

Declaration of interests

R.J.M., J.E.H., and E.R.P. are co-founders, scientific advisors, and hold equity in Dynomics, a biotechnology company focused on the development of heart failure therapeutics. P.F. and P.G.V.M. are employees of and hold equity in Moderna.

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