Cell type-selective targeted delivery of a recombinant lysosomal enzyme for enzyme therapies

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Lysosomal diseases are a class of genetic disorders predominantly caused by loss of lysosomal hydrolases, leading to lysosomal and cellular dysfunction. Enzyme replacement therapy (ERT), where recombinant enzyme is given intravenously, internalized by cells, and trafficked to the lysosome, has been applied to treat several lysosomal diseases. However, current ERT regimens do not correct disease phenotypes in all affected organs because the biodistribution of enzyme uptake does not match that of the affected cells that require the enzyme. We present here targeted ERT, an approach that utilizes antibody-enzyme fusion proteins to target the enzyme to specific cell types. The antibody moiety recognizes transmembrane proteins involved in lysosomal trafficking and that are also preferentially expressed in those cells most affected in disease. Using Pompe disease (PD) as an example, we show that targeted ERT is superior to ERT in treating the skeletal muscle phenotypes of PD mice both as a protein replacement therapeutic and as a gene therapy.

INTRODUCTION

Pompe disease (PD), or glycogen storage disease type II, is a monogenic, lysosomal disease caused by a deficiency in the activity of the enzyme lysosomal acid alpha-glucosidase (GAA). GAA deficiency results in an accumulation of its substrate, glycogen, in the lysosomes of cells in tissues including skeletal and cardiac muscle. This aberrant accumulation of glycogen in myofibers results in progressive damage of muscle tissue, with symptoms that can include cardiomegaly, mild to profound muscle weakness, and ultimately death due to cardiac or respiratory failure1. Inadequate delivery of rhGAA to skeletal muscle has been suggested as the main reason ERT fails to treat this organ system. The primary mechanism by which rhGAA reaches lysosomes is through uptake by the cation-independent mannose 6-phosphate (M6P) receptor (CI-MPR), which binds M6P on rhGAA. However, CI-MPR expression in skeletal muscle is very low, and rhGAA is poorly mannose 6-phosphorylated.6,7 In addition, CI-MPR may be misdirected into autophagosomes in affected cells, rather than lysosomes,8 while a large amount of the drug is also taken up by the liver (an organ that does not have primary pathology in PD). To overcome these problems, rhGAA is given at a high and frequent dosing (20–40 mg/kg once every 2 weeks); nonetheless, difficulties in treating skeletal muscle persist. Strategies to modify rhGAA to increase delivery to muscle by manipulating the affinity of GAA to CI-MPR, such as increasing the M6P content of GAA9 or using the IGF2 binding sites on CI-MPR10 have been explored in the clinic. Still, these methods fail to address the underlying inherent limitations of using CI-MPR as the mechanism for uptake, i.e., CI-MPR’s low level of muscle expression and its largely endosomal rather than surface localization in skeletal muscle, which, combined with high expression in the liver, result in very poor uptake by muscle.11,12

As is the case for several other lysosomal diseases, PD is currently treated by enzyme replacement therapy (ERT). For PD, recombinant human (rh) GAA is delivered by intravenous infusion into patients every other week. While ERT has been very successful in treating the cardiac manifestations of PD, skeletal muscle and the central nervous system (CNS) remain minimally treated by ERT.2–4

Adeno-associated virus (AAV)-based liver depot gene therapies have shown promise to allow for continuous-dosing ERT by infecting hepatocytes in vivo to produce and secrete GAA.13,14 Additionally, liver depot gene therapy of GAA has been shown to tolerate GAA knockout (KO) mice to GAA and mitigate the antibody immune response to GAA in the serum.15,16 However, while the immune response was dampened, correction of skeletal muscle glycogen accumulation was
only achieved at very high serum levels of GAA,17 likely a reflection of the continued reliance on CI-MPR for uptake by skeletal muscle. It was also unclear what proportion of hepatically secreted GAA is mannose 6-phosphorylated, as overexpression of GAA in other cell systems has been shown to adversely affect M6P content of GAA.6,18

Therefore, the current state of the art is one of only incremental progress despite continued attempts at improving ERT. Using PD as an example, we present here targeted ERT, a new technology that addresses the delivery and biodistribution limitations of ERT by employing antibody-enzyme fusion proteins as the drug and testing them in PD mice. In targeted ERT, the antibody moiety targets the enzyme, GAA, to the affected cells by binding to transmembrane proteins that are both trafficked to the lysosome and preferentially expressed on the cell type of interest, thereby achieving superior clearance of the substrate—glycogen—compared to non-targeted GAA. We demonstrate the superior efficacy of this technology by comparing purified targeted GAA protein to GAA alone. We also adapt targeted ERT to gene therapy, using AAV-mediated gene delivery to the liver, in order to provide long-term efficacy while also alleviating the need for repeated dosing. This positions targeted ERT as a potential modality that can be adopted in emerging gene therapy-based treatments for lysosomal diseases (LDs).

RESULTS
Antibody:GAA fusion proteins display enzymatic activity equivalent to GAA

In order to address the delivery limitations associated with rhGAA ERT—i.e., loss of drug to the liver and poor delivery to skeletal muscle—we chose to bypass CI-MPR-mediated delivery of GAA and explore antibody-enzyme fusion proteins as a means to target the enzyme to the affected cells and tissues. These fusion proteins have to display the following properties:

1. Retain enzymatic function.
2. Bind to effector proteins that traffic to the lysosome, most likely transmembrane or cell surface proteins that are also preferentially expressed in the cells most affected in disease, while displaying minimal or no expression in non-affected cells and acting independently of CI-MPR.

In order to identify proteins with the desired properties specifically for GAA ERT, we screened an internal database of gene expression profiles and defined a set of transmembrane and cell surface proteins that are expressed in skeletal muscles, and which are minimally expressed in the liver. We then looked among them for those with monoclonal antibodies readily available. Two of these proteins, CD63 (Figures S1A, S1B, and Supplemental Methods) and ITGA7,19 met all three criteria. Furthermore, CD63 was already known to traffic between the cell surface and lysosomes,20,21 suggesting that it could be used as a benchmark to compare other potential effectors.

Starting with CD63, we engineered antibody:GAA fusions where the C terminus of full-length immunoglobulin G (IgG)4 antibody or a single-chain fragment variable (scFv) was fused to the N terminus of amino acids 70–952 of GAA with a glycine-serine linker (Figure S1C), expressed, and purified. The enzymatic activity of the IgG:GAA or scFv:GAA purified fusion proteins was found to be comparable to alglucosidase alfa (recombinant GAA). Three different anti-CD63 variable domains were used, one being derived from immunized wild-type mice (denoted as α-hCD631), one derived from immunized mice that produce human antibodies (denoted as α-hCD632),22 and finally an anti-mouse CD63 variable domain derived from rat (denoted as α-mCD63). The binding dissociation constant (Kd) between the two human antibody variable domains to the extracellular loop 2 of hCD63 was found to be in the nanomolar to subnanomolar range (Figure S1E). The Kd of the anti-hCD632 antibody was also measured when formatted into IgG:GAA and scFv:GAA formats (diagrams in Figure S1C), and the binding affinity to hCD63 remained in the nanomolar range.

Internalization of antibody:GAA fusions is an antibody-dependent, CI-MPR-independent process

To verify the antibody-mediated properties of the antibody:GAA fusions, the α-hCD63, IgG:GAA fusion was incubated with HEK293 cells overnight to allow for intracellular uptake. Dose-dependent cellular uptake was observed, while a non-binding antibody:GAA control showed no uptake in these cells. Furthermore, α-hCD63, IgG:GAA did not internalize in HEK293 cells that are deficient for hCD63. These results demonstrated that α-hCD63, IgG:GAA internalized specifically through the effector (CD63) recognized by the antibody domain of the fusion protein (Figure 1A). α-hCD63, IgG:GAA was also assayed for internalization in human primary myoblasts (Figure 1B). Uptake saturated at ~50 nM and was not inhibited by the presence of 5 mM M6P, a competitive inhibitor for CI-MPR binding. The ability of 5 mM M6P to inhibit CI-MPR-mediated uptake of GAA was verified in the same cell line. A glycan analysis (Table S1 and Supplemental methods) of α-hCD63, IgG:GAA showed that the glycan structures were mainly terminally sialylated and mannosylated, and terminal M6P was not detected on any N-glycan sites of GAA.

To explore whether the properties observed with α-hCD63:GAA are shared when other effectors are utilized, we tested an α-ITGA7 IgG:GAA fusion protein. This targets mouse integrin alpha-7, a surface protein preferentially expressed in muscle cells.23 Purified α-ITGA7 IgG:GAA protein was incubated with C2C12 mouse myoblasts overnight and compared to an α-mCD63 IgG:GAA. Both constructs were internalized in C2C12 myoblasts independently of M6P, as 5 mM M6P did not decrease uptake (Figure 1C). However, α-mCD63 IgG:GAA exhibited higher internalization than α-ITGA7:GAA.

Antibody:GAA fusions undergo normal intracellular processing of GAA

To determine whether the precursor GAA on the α-hCD63 IgG:GAA fusions retains the intracellular trafficking and proteolytic processing events to form mature lysosomal forms of GAA,
especially given that the GAA fusion preparations lacked the canonical M6P residues for trafficking via CI-MPR, we performed a pulse-chase experiment of α-hCD63 IgG:GAA on a Pompe patient fibroblast line that lacks the lysosomal GAA forms. Within 24 h of internalization, accumulation of the 76-kDa lysosomal form of GAA was observed (Figure 1D). The half-life of the lysosomal GAA matched the long intracellular half-life of other rhGAA preparations, while the antibody portion of the fusion quickly degraded over 3 days.

Cathepsin inhibitors known to abolish the processing of GAA to the lysosomal form prevented full processing of the IgG:GAA to the lysosomal 76-kDa form (Figure S2), further indicating that the α-hCD63 IgG:GAA fusion protein is converted in the lysosome into mature GAA.

scFv:GAA fusions also mediate uptake and processing of GAA

We then tested whether an scFv:GAA format was able to achieve similar uptake and processing as an IgG:GAA format, as the smaller transgene size would allow for packaging into recombinant AAV genomes for gene therapy. α-hCD63 scFv:GAA or alglucosidase alfa was incubated with HEK293 cells overnight, and the scFv:GAA format, like alglucosidase alfa, also accumulated the 76-kDa form of lysosomal GAA by western blot in HEK cells in a dose-dependent fashion (Figure 2A). We also observed that internalized α-hCD63 scFv:GAA co-localized with LAMP2, a lysosomal marker, similarly to internalized alglucosidase alfa (Figure 2B). Additionally, a glycan analysis of the purified α-hCD63 scFv:GAA protein demonstrated primarily sialylated and mannosylated N-glycan structures with no detectable terminal M6P residues, similar to the α-hCD63 IgG:GAA protein (Table S1).

Targeted GAA has superior in vivo efficacy in PD mice versus GAA alone when administered as a protein ERT

The relative efficacy of targeting GAA using α-hCD63 compared to the standard of care, alglucosidase alfa, was tested in a mouse model of PD. Given that these fusion proteins recognize human but not mouse CD63, we first engineered a PD mouse model, Gaa<sup>+/-</sup>;Cd63hu/hu, where Gaa was replaced by LacZ and the protein-coding region of the Cd63 locus was replaced with its human counterpart (Figures S3A and S3B). Gaa<sup>+/-</sup>;Cd63hu/hu mice displayed levels of glycogen storage equivalent to a previous Gaa<sup>6neo/6neo</sup> mouse model (Figure S3C).<sup>26</sup> Alglucosidase alfa or α-hCD63scFv:GAA was then dosed intravenously weekly for 4 weeks in 2- to 3-month-old PD mice. Mice were sacrificed 1 week after the last dose. Alglucosidase alfa was given at a dose of 5 mg/kg or 20 mg/kg, and α-hCD63scFv:GAA were given at the respective equimolar doses of 6.2 mg/kg or 25 mg/kg, respectively. A non-depleting anti-CD4 was administered intraperitoneally 1 day prior to each weekly injection to all groups to mitigate anaphylactic and anti-drug reactions to the test molecules.<sup>27</sup>

Glycogen levels were quantified in muscle tissue lysates of the sacrificed animals (Figure 3A). In both low- and high-dose groups, α-hCD63scFv:GAA removed more glycogen compared to alglucosidase alfa (p < 0.01 in all tissues measured between equivalent molar doses). Furthermore, there was a dose-dependent decrease of glycogen, with 25 mg/kg of α-hCD63scFv:GAA clearing more glycogen than the 6.2 mg/kg dose (p < 0.01).
Quadriceps from untreated PD mice showed diffuse staining of LAMP1 lysosomes that were present throughout the muscle fibers rather than restricted mainly to the periphery of the fiber as in wild-type controls (Gaa+/+; Cd63hu/hu) (Figure 3B). Treatment with α-hCD631 scFv:GAA reduced lysosomal staining throughout the muscle fibers at the higher 25 mg/kg dose.

**AAV-mediated liver depot gene therapy of α-hCD63 scFv:GAA clears muscle glycogen to wild-type levels**

In order to achieve long-term expression and sustained delivery of targeted GAA proteins, we utilized AAV-mediated liver depot gene therapy in our PD mouse model. AAV2/8 viruses encoding α-hCD63 scFv:GAA or GAA cDNAs driven by a liver-specific promoter were injected by tail vein into 2- to 3-month-old PD mice at 1e10 or 1e11 viral genomes (vg)/mouse. Serum levels of GAA proteins were higher in the AAV α-hCD63 scFv:GAA groups than in the AAV GAA-treated groups at equivalent doses (Figure 4A), possibly because of inefficient secretion mediated by the native GAA signal peptide.13 AAV-infected PD mice were sacrificed after 3 months, and tissues were assayed for glycogen. Qualitatively, clearance of glycogen to wild-type levels was observed in periodic acid-Schiff (PAS)-stained sections of quadriceps, and liver 3 months after infection, and expression was predominant in the liver for all constructs in a viral dose-dependent fashion (Figure 4A).

Increased autophagy is well documented in PD and in PD mice8,28 and may be a key part of the mechanism of pathogenesis. LC3b is a protein component of the autophagosome and a commonly used marker of autophagy. To examine autophagy in skeletal muscle, western blotting for LC3b was carried out on quadriceps lysates from the AAV GAA-treated group was not significantly different from control mice (Figure 5A). Treatment with AAV α-hCD63 scFv:GAA improved lysosomal localization (Figure 5A) and significantly reduced the total lysosomal area (Figures 5B and 5C). In contrast, treatment with AAV GAA showed a trend toward reduced lysosomal area, but the difference from the untreated group was not significant.

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Treatment with AAV \( \alpha \)-hCD63 scFv:GAA significantly reduced both LC3b-I and LC3b-II, while treatment with AAV GAA had a much smaller effect (Figures 5D–5F).

Rotarod and grip strength measurements improved in GAA KO mice after treatment with AAV \( \alpha \)-hCD631 scFv:GAA

To assess whether the improved glycogen reduction seen with AAV \( \alpha \)-hCD631 scFv:GAA translated into improved muscle function, 2- to 3-month-old PD mice were either treated with a single dose of 1e11 vg/mouse of AAV \( \alpha \)-hCD631 scFv:GAA or AAV GAA or left untreated as controls. Age-matched Gaa\(^{+/+}\);Cd63hu/hu served as wild-type controls. The mice were tested on Rotarod and grip strength apparatuses 1 week prior to AAV administration and then monthly for the following 6 months.

Mice treated with AAV \( \alpha \)-hCD631 scFv:GAA began showing significantly improved performance compared to untreated mice on both the grip strength and Rotarod tests at 2 and 3 months after AAV administration, respectively (Figures 6A and 6B). On both measures, performance of AAV \( \alpha \)-hCD631 scFv:GAA-treated mice tracked closely with that of wild-type mice from 3 months of treatment on.

Starting at 3 months after treatment, the performance of mice treated with AAV \( \alpha \)-hCD631 scFv:GAA on Rotarod was also significantly better than mice treated with AAV GAA. Mice treated with AAV GAA showed a small trend toward improvement in both measures, but it was not statistically significantly different from untreated mice.

The tibialis anterior (TA) muscles of mice 6 months after treatment of AAV \( \alpha \)-hCD631 scFv:GAA were excised, and the peak tetanic force was measured compared to untreated and wild-type control mice (Figure 6C). Both AAV \( \alpha \)-hCD631 scFv:GAA and wild-type mouse muscle had higher peak forces, and the AAV \( \alpha \)-hCD631 scFv:GAA-treated muscle was not significantly different from wild-type mice, suggesting a recovery in muscle strength from the treatment.

Modest reductions in CNS glycogen after AAV treatment

CNS tissues were assayed for glycogen removal 3 and 10 months after treatment with the high 1e11 vg/mouse dose. Trends of reduction were seen in both AAV \( \alpha \)-hCD631 scFv:GAA- and AAV GAA-treated groups, but the only significant decrease in glycogen levels was in the spinal cord in the AAV \( \alpha \)-hCD631 scFv:GAA-treated mice after 10 months of treatment (Figure S5).
Serum level threshold of \(a\)-hCD63 scFv to normalize glycogen levels in skeletal muscle

Using human antibodies for the antibody portion of the scFv:GAA fusion would most likely mitigate immunogenicity of the scFv portion of the molecule in patients. As a bridging study between the mouse \(a\)-hCD63 and human \(a\)-hCD63 variable domains, we compared glycogen removal from a 1e11 vg/mouse dose after 3 months of treatment of AAV \(a\)-hCD63 scFv:GAA and AAV \(a\)-hCD63 scFv:GAA. Both \(a\)-hCD63 and \(a\)-hCD63 scFv:GAA constructs were able to mediate similar levels of glycogen clearance (Figure S6 and Supplemental methods), and \(a\)-hCD63 scFv:GAA normalized the heart wet weight to wild-type levels after 1 month of treatment (Figure S7).

To better quantify the level of \(a\)-hCD63 scFv:GAA in the serum, we developed an ELISA to detect the scFv:GAA. One month after AAV administration, in mice where the serum level of \(a\)-hCD63 scFv:GAA was >5 \(\mu\)g/mL, glycogen levels were normalized to wild-type levels (Figure S8). In comparison, >50 \(\mu\)g/mL serum GAA is required for similar levels of glycogen clearance in PD mice.15 It is important to note, though, that \(a\)-hCD63 scFv:GAA and GAA may have different rates of clearance from the serum given their different uptake mechanisms, so an equivalent steady-state serum level of \(a\)-hCD63 scFv:GAA and GAA does not necessarily indicate that equivalent numbers of molecules of \(a\)-hCD63 scFv:GAA and GAA are being produced.

DISCUSSION

We report here targeted ERT, a method for engineering lysosomal enzymes in a manner that enhances their delivery to the cells where they are needed. Targeted ERT achieves this goal by fusing the enzyme to antibodies that bind transmembrane or cell surface proteins—“effector proteins”—that traffic to lysosomes and are preferably expressed in the cell types affected in the corresponding lysosomal disease. Using PD as an example, we focused on delivery of GAA in PD mice and chose antibodies against effector proteins that are enriched in cardiac and skeletal muscle, the primary targets for PD therapy. We demonstrate that targeted delivery of GAA is highly effective in the mouse PD model and circumvents the limitations of the CI-MPR-mediated delivery.

Conceptually, any transmembrane or cell surface protein that is expressed in “disease-relevant” cells and internalizes to the lysosome can potentially be used as an effector protein, provided that it is
Sufficiently abundant and that an antibody can be raised against it. In this study, two different types of effector proteins were chosen to test targeted ERT in PD. The first was CD63, a tetraspanin with broad expression across many cell types yet highly expressed in skeletal muscle, and the second was ITGA7, an integrin that is enriched in skeletal and cardiac muscle.

\(\alpha\)-hCD63 scFv:GAA showed better uptake in vitro compared to untargeted GAA, and showed improved glycogen removal in PD mice. Furthermore, using gene therapy as the mode of delivery, we have achieved improved muscle function in a longitudinal study. While initial results are promising when the treatment is delivered to young mice, further tests should be performed to determine if a targeted enzyme delivery system could address the decreased efficacy of conventional ERT in older PD mice. Glycogen clearance in mice treated with recombinant GAA late in the course of disease was not sufficient to recover motor function or reverse muscle damage. Insufficient CI-MPR-mediated delivery, while significant, may play a much smaller role than the inability of muscle to repair severely damaged fibers in aged mice.

Additionally, recent evidence suggests a role for glycogen accumulation in tissues other than cardiac and skeletal muscle in the pathology of PD. Abnormalities in the neuromuscular junction have been linked with glycogen deposits in spinal motor neurons in mice, and infantile-onset patients show secondary symptoms indicative of neural involvement. Studies normalizing glycogen levels solely in the brain and CNS in PD mice (using AAV gene therapy) have shown correction of some neuromuscular phenotypes even in the absence of improvement in cardiac or skeletal muscle fibers, demonstrating that a full reversion of clinical phenotypes in PD ERT will require multisystem delivery. While we and others have seen a trend for partial reduction in CNS glycogen storage, it remains unknown.

**Figure 5.** Treatment of PD mice with AAV \(\alpha\)-hCD63 scFv:GAA reduces lysosomal area and autophagy

(A and B) Confocal images (A) and wide-field images (B) of anti-LAMP1 staining to detect lysosomes in section of quadriceps in mice 3 months after administration of AAV GAA or \(\alpha\)-hCD63 scFv:GAA show that lysosome staining is decreased with scFv:GAA treatment. Scale bars, 100 \(\mu\)m. (C) Quantification of the LAMP-1-positive area in wide-field images. n = 2 for wild type, n = 4 for treatment groups. Error bars represent means ± SD. (D) Autophagy is decreased with \(\alpha\)-hCD63 scFv:GAA treatment. Representative western blotting for LC3b to monitor autophagy in quadriceps lysate from two mice per group. (E and F) Quantification of LC3b-II (E) and LC3b-I (F) levels in quadriceps lysates. n = 2 for wild type, n = 4 for treatment groups. Error bars represent means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.
whether this reduction will be clinically significant in PD patients. To maximize enzyme delivery to affected cell and tissue types, an effector like CD63, which—in addition to cardiac and skeletal muscles—is also expressed in the CNS, would likely be more appropriate than more tissue-restricted internalizers like ITGA7. Therefore, adding blood-brain barrier crossing functionality to neuronal lysosome targeting in a format such as a bispecific antibody may be able to improve overall efficacy.33,34 Alternatively, AAV may be delivered via an intrathecal or intracerebroventricular route as well as intravenously to treat both the CNS and skeletal muscle.31,32

Figure 6. Treatment of PD mice with AAV α-hCD63, scFv:GAA restores muscle function in PD mice
(A) Rotarod test performance of mice treated with either AAV GAA or AAV α-hCD63, scFv:GAA shows recovery of scFv:GAA-treated mice within 2 months of AAV administration. (B) Forelimb grip strength measurements show continued strength improvement within 1 month of treatment in α-hCD63:scFv;GAA-treated mice. PD mice were treated with 1e11 vg AAV α-hCD63, scFv:GAA or 1e11 vg AAV GAA or were left untreated, and these groups were compared to wild-type mice. (C) Ex vivo peak tetanic force of the tibialis anterior muscle 6 months after AAV treatment shows a recovery in muscle strength in the AAV α-hCD63, scFv:GAA-treated group to comparable to wild-type levels. (A and B) Error bars represent medians ± SD, n = 7 for wild type, n = 11 for treatment groups. (C) Error bars represent means ± SD, n = 4 for untreated and wild type, n = 5 for treatment group. *p < 0.05, **p < 0.01, ***p < 0.001.

ERTs for other lysosomal diseases, such as rhGALNS for mucopolysaccharidosis VIA and rhARSB for mucopolysaccharidosis VI, also have poor delivery to diseased non-CNS organs partially due to the limitations of CI-MPR.35 Enzyme delivery to address the skeletal phenotypes in mucopolysaccharidoses I–VII remains a challenge because of the virtually nonexistent delivery to chondrocytes and cartilage tissue.36 Antibody targeting of these enzymes in a manner similar to that presented here using internalizers enriched in growth plate or articular chondrocytes may be able to enhance enzyme delivery and correction of the skeletal phenotypes.

One challenge in the selection of an appropriate targeting effector protein is the potential for targeting antibody to disrupt the normal function of the effector protein. A careful selection of the antibody such that it does not influence function (e.g., activation, inhibition, or degradation) of the effector protein is warranted. Moreover, effectors that are unlikely to cause negative outcomes even if their function is affected should be considered. In an evaluation of CD63 as an antibody target, it is known that Cdh6 KO mice are fully viable and effectively normal.37 Only some mild renal phenotypes and leukocyte defects have been noted in Cdh6 KO mice. A more comprehensive toxicology panel in higher animal models is warranted to investigate any possible side effects of using CD63 as an effector for safety and toxicity.

The approach to clinical development of targeted ERT will be determined by the therapeutic format, as targeted ERT can be administered as either a purified protein or a gene therapy. Infusion of a purified protein should be considered because of its maturity as a therapeutic modality, whereas a gene therapy approach may offer several potential advantages. An AAV approach alleviates the need for every other
week infusions of ERT. However, liver-targeted AAV-based ERT is unlikely to lead to lifelong expression of the transgene because of hepatocyte turnover, especially in younger patients. Furthermore, neutralizing antibodies (nAbs) against the AAV capsids may not only exclude patients from treatment eligibility but further prevent re-dosing AAV therapies, although immunomodulation and tolerization approaches are currently under study that may show a path forward to overcome nAbs against AAV capsids. Another potential advantage of the AAV approach is that it may help overcome nAbs against the replacement enzyme. An immune response to rhGAA is known to occur in a significant number of PD patients and often requires significant clinical intervention. Immunogenicity of the replacement enzyme still remains a major concern with targeted ERT, and immunogenicity may even be enhanced if the antibody drives the fusion protein to antigen-presenting cells or if there are neo-epitopes in the scFv junctions of the fusion protein. Interestingly, promising evidence for the ability of liver depot gene therapy to rects the fusion protein to antigen-presenting cells or if there are neo-epitopes in the fusion protein. Interestingly, promising evidence for the ability of liver depot gene therapy to reverse pre-existing anti-drug antibodies has been shown in mice. Clinical trials to test whether this is true in humans have already begun. Finally, it should be noted that the modularity and interchangeability of the targeting antibody is an inherent advantage to this platform technology over delivery technologies that utilize a single binding domain/receptor interaction. Swapping (or even combining) targeting antibodies to different protein targets to tune delivery to desired cell types while avoiding other cell types may turn out to be a significant advance in the field of enzyme therapies.

MATERIALS AND METHODS

Plasmid backbones for protein production, HDD, and AAV

Antibody-GAA plasmid constructs were created by isothermal assembly. IgG:GAA fusion constructs were cloned with a S228P hinge stabilized human IgG4 antibody (Uniprot P01861) backbone with an amino acid linker comprising GGGGS and amino acids 70–952 of human GAA (GenBank: NM_000152.4). Full-length human GAA cDNA (amino acids 1–952) plasmids were used as a comparator. AAV plasmids used a liver-specific transthyretin promoter with a liver enhancer from Serpinat. scFv fusions were cloned with domains in the following order: Vh, 3 × glycine-serine linker, Vk, 1 × glycine-serine linker, amino acids 70–952 of human GAA. ζ-hCD63; scFv:GAA was deposited as GenBank: MZ683483.1, and ζ-hCD63; scFv:GAA was deposited as GenBank: MZ683484.1.

Antibody variable domains

Anti-human CD63 antibodies and their fusions used either the H5C6 mouse anti-human CD63 variable domains (denoted here as ζ-hCD63; ) (Iowa Developmental Studies Hybридoma Bank) or a fully human anti-human CD63 variable domain derived from a Velocimmune mouse technology (denoted here as ζ-hCD63; ). Anti-mouse integrin alpha-7 antibodies and their fusions used the variable domains of mouse anti-mouse integrin alpha-7 non-blocking antibody 3C12.

GAA and antibody-GAA proteins

Antibody-GAA constructs were transfected into Chinese hamster ovary (CHO) cells and purified by protein A or protein L chromatography. Briefly, CHO supernatants were flowed through protein L or protein A columns (Cytiva, Marlborough, MA, USA) in 50 mM Tris, pH 7.5, 150 mM NaCl. Columns were washed with 50 mM Tris, pH 7.5, 500 mM NaCl. Proteins were eluted with IgG Elution Buffer at pH 2.8 (Thermo Fisher, Waltham, MA, USA) and neutralized with 1 M Tris, pH 8.5. The protein solution was buffer exchanged to 20 mM sodium phosphate, 150 mM NaCl, pH 6.2 by dialysis. Size-exclusion chromatography on Superose 6 PGS was used to remove high-molecular-weight contaminants. Final product was stored in 20 mM sodium phosphate, 150 mM NaCl, pH 6.2. For some experiments rhGAA was expressed in CHO-K1 cells by transiently transfecting a plasmid encoding an mROR signal peptide with a myc tag (EQKLISEEDL) followed by amino acids 70–952 of human GAA. Supernatants were collected 72 h after transfection, bound on a myc column (Thermo Fisher, Waltham, MA, USA), and eluted with myc peptide (Thermo Fisher, Waltham, MA, USA). Experiments using alglucosidase alfa (clinical-grade rhGAA) were prepared according to manufacturer’s dilution instructions (Sanofi Genzyme, Boston, MA, USA). Enzyme activity was measured on freshly prepared alglucosidase alfa. In vivo experiments used flash-frozen aliquots of alglucosidase alfa.

In vitro internalization of antibody-GAA constructs

Primary adult human myoblasts (Lonza CC-2561, Switzerland), infantile Pompe fibroblast lines GM20089 and GM20090 (Coriell, Camden, NJ, USA), C2C12 myoblasts, and CRISPR-modified HEK293 cells were used to test internalization of antibody-GAA constructs. GM20089 fibroblasts are missing exon 18 of GAA, and GM20091 fibroblasts are a compound heterozygote of Q58X and del525T. Both lines are deficient in processing of GAA pre-protein to its mature lysosomal form. Antibody:GAA proteins were incubated with semi-confluent cells overnight, washed extensively with PBS, and then lysed with either radioimmunoprecipitation assay (RIPA) buffer for protein analyses or sodium acetate lysis buffer (0.2 mM sodium acetate, 0.4 mM potassium chloride, 0.5% NP-40, pH 4.3). Cells were incubated with leupeptin or E-64 (Sigma-Aldrich, St. Louis, MO, USA) in experiments testing GAA intracellular processing and cleavage age. For pulse-chase experiments, cells were incubated overnight with a dose of 50 nM ζ-hCD63; GAA in the medium. After incubation, cells were washed once with PBS and given fresh medium. Medium was changed every 3 days over the course of the experiment.

Generation of CD63-deficient HEK293 cells by CRISPR

To generate CD63-deficient HEK293 cells, cells were co-transfected using LTI reagent and protocol (Mirus Bio, Madison, WI, USA) with a plasmid containing Cas9+ NLS under the cytomegalovirus (CMV) promoter and a plasmid containing the guide RNA CACT CACGCAAAGGGCCAGG under the U6 promoter. Pooled transfected cells were then trypsinized, stained with ζ-hCD63 antibody H5C6 (Developmental Studies Hybридoma Bank, Iowa City, IA, USA) and goat anti-mouse Alexa647 (Thermo Fisher, Waltham, MA, USA), and then subjected to fluorescence-activated cell sorting (FACS). The cell population negative for anti-hCD63 staining was collected and expanded. The pool was then further confirmed to
have uniform negative staining for CD63 by flow cytometry using the same antibodies.

**GAA knockout mouse models**

Mouse experiments were performed with the approval of the Regeneron Institutional Animal Care and Use Committee. All experiments conformed to federal and state laws regarding animal research. The GAA<sup爿<sub>128911372-128912754</sub>–<sub>128912754</sub></sup> mouse line<sup>45,46</sup> was obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Cd63 humanized, Gaa homozgyous null (Cd63<sub>hu/hu</sub> Gaa<sup>−/−</sup>) mice were generated with the VelociGene method.<sup>45,46</sup> Nucleotide coordinates corresponding to those in mouse genome Ensembl release 98 (September 2019) are provided to facilitate a precise description of the alleles generated. To begin, an allele with fully humanized Cd63 coding sequence was generated via recombinetiong technology. Mouse genome sequence with coordinates 128911372–128912754 on chromosome 10, which is contained with BAC RP23-122115, was replaced with the Human genome coordinates 55725292–55727639 from chromosome 12. This replaces mouse Cd63 exons 2–7 (ENSMUSE00000150030–ENSMUSE00001408024) with the orthologous human CD63 exons, part of the 1<sup>st</sup> intron, intervening introns, and 3<sup>′</sup> non-coding sequences. Exon 1 (ENSMUSE00000150024) was not replaced as it encodes protein sequence 100% identical to human. A self-deleting, fLoxed hygromycin resistance cassette was placed in the intron following human coding exon 5 (ENSE00003590609) between coordinates 12:55729738 and 55728596. The self-deleting cassette contained the hygromycin resistance coding sequence under the control of the human UBC promoter and an Em7 promoter for growth in *E. coli*, followed by a poly(A) signal from mouse Pkg1. For deletion in the F0 male germline, the cassette also contained Cre coding sequence controlled by the mouse Prm1 promoter and an SV40 poly(A) signal. The Cre coding sequence was interrupted by a synthetic intron, to prevent expression in bacteria. The resulting targeting construct had 125- and 84-kb homology arms, respectively, and contained a hybrid Cd63/CD63 allele encoding protein 100% identical to human CD63. A GenBank file with the sequence of the modified allele is supplied in the Supplemental information as Supplemental Sequence 1. The targeting construct was then linearized with NotI and electroporated into Cd63<sup>−/−</sub> hyg<sup>+</sup> embryonic stem cells. After microinjection, F<sub>0</sub> offspring were bred to wild-type C57BL/6NTac and genotyped for alleles VG7233 (Cd63<sub>hu/hu</sub>), and VG6533 (Gaa<sup>−/−</sup>), resulting from male germline deletion of the hygromycin and neomycin cassettes, respectively. Animals were then intercrossed to generate Gaa<sup>−/−</sup>; Cd63<sub>hu/hu</sub> cohorts used for subsequent experiments.

**In vivo purified protein ERT**

Two- to three-month old PD mice (Gaa<sup>−/−</sup>; Cd63<sub>hu/hu</sub>) were dosed weekly with 5 mg/kg alglucosidase alfa, 20 mg/kg alglucosidase alfa, 6.2 mg/kg α-CD63<sub>2</sub> scFv:GAA, or 25 mg/kg α-CD63<sub>2</sub> scFv:GAA. To mitigate anaphylactic reactions, all mice were dosed with intraperitoneal 50 mg/kg anti-C4D (YTS177, Bio X Cell, Lebanon, NH, USA) 1 day prior to each injection of alglucosidase alfa or α-CD63<sub>2</sub> scFv:GAA. Mice were injected intravenously weekly for 4 weeks and sacrificed 1 week after the last dose. Tissues for glycogen quantification were dissected, snap frozen, and stored at −80°C prior to running the assays.

**AAV production and in vivo transduction**

Recombinant AAV8 (AAV2/8) was produced in HEK293 cells. Cells were transfected with three plasmids encoding adenovirus helper genes, AAV8 rep and cap genes, and recombinant AAV genomes containing transgenes flanked by AAV2 inverted terminal repeats (ITRs). On day 5, cells and medium were collected, centrifuged, and processed for AAV purification. Cell pellets were lysed by freeze-thaw and cleared by centrifugation. Processed cell lysates and medium were overlaid onto iodixanol gradients columns and centrifuged in an ultracentrifuge. Virus fractions were removed from the interface between the 40% and 60% iodixanol gradients columns and centrifuged in an ultracentrifuge. Virus fractions were removed from the interface between the 40% and 60% iodixanol solutions and exchanged into 1× PBS with desalting columns. AAV vg were quantified by qPCR using TaqMan oligos targeting the ITR. A standard curve was generated using serial dilutions of virus with a known concentration. AAVs were diluted in PBS + 0.001% F-68 Pluronic immediately prior to injection.

**Muscle performance assays**

AAV-treated mice were subjected to monthly Rotarod and forelimb grip strength measurements. The Rotarod (ITC Life Sciences, Woodland Hills, CA, USA) was programmed to accelerate at 0.5 rpm/s for 60 s, and time to fall was recorded. For the grip strength testing, mice were placed on a flat wire mesh, and the mesh was inverted. Time to fall was recorded with a maximum of 60 s. Forelimb grip strength was measured with a force meter (Columbus Instruments, Columbus, OH, USA). All tests were performed in triplicate at each time point.

**Ex vivo muscle physiology**

To determine whether AAV α-hCD63<sub>1</sub> scFv:GAA treatment affected TA muscle function, *ex vivo* force was measured 6 months after dosing as previously described.<sup>47</sup> Briefly, TA muscles were removed from anesthetized Pompe untreated, AAV α-hCD63<sub>1</sub> scFv:GAA, and GAA wild-type mice. Maximal twitch force and peak isometric tetanic force were measured *ex vivo* immediately after dissection.
**Tissue collection and glycogen measurements**

Tissues were dissected from mice immediately after sacrifice by CO2 asphyxiation, snap frozen in liquid nitrogen, and stored at −80°C. Tissues were lysed on a benchtop homogenizer with stainless steel beads in distilled water for glycogen measurements or RIPA buffer for protein analyses. Glycogen analysis lysates were boiled and centrifuged to clear debris. Glycogen measurements were performed fluorometrically with a commercial kit according to manufacturer’s instructions (K646, BioVision, Milpitas, CA, USA).

**Histology**

Mouse tissues were embedded immediately after dissection in optimal cutting temperature compound (Tissue-Tek O.C.T.) chilled in a bath of isopentane chilled in a bath of liquid nitrogen. Blocks were stored at −80°C until they were cut at 12 μm and mounted onto slides. Sectioning and PAS-II staining was performed by Histoserv (Germantown, MD, USA). For antibody staining, slides were post-fixed in 4% paraformaldehyde and blocked in eBioscience Blocking Buffer (00-4959-52, Thermo Fisher, Waltham, MA, USA), followed by staining with anti-Lamp1 1D4B (ab25245, Abcam, Cambridge, MA, USA). For fluorescent imaging, slides were then stained with goat anti-Rat Alexa555 (A21434, Thermo Fisher, Waltham, MA, USA), mounted in Fluoromount-G with DAPI (00-4959-52, Thermo Fisher, Waltham, MA, USA), and imaged with a Zeiss LSM 710. For visible light imaging, slides were quenched with 30% hydrogen peroxide prior to blocking as above after the primary antibody, then treated with biotinylated donkey anti-rat IgG (A18743, Thermo Fisher, Waltham, MA, USA) followed by 3,3’-diaminobenzidine (DAB) staining with an ABC kit (PK-6100, Vector Labs, Burlingame, CA, USA) and DAB (D5637, Sigma-Aldrich, St. Louis, MO, USA). Slides were then dehydrated in alcohols, cleared in xylene, coverslipped, and imaged on an Aperio slide scanner. Images were quantified with HALO software.

**Western blots**

Cell and tissue lysates were prepared by lysis in RIPA buffer with protease inhibitors (1861282, Thermo Fisher, Waltham, MA, USA). Tissue lysates were homogenized with a bead homogenizer (Fast-Prep5, MP Biomedicals, Santa Ana, CA, USA). Cells or tissue lysates were run on SDS-PAGE gels using the Novex system (LifeTech Thermo, XPO4200BOX, LC2675, LC3675, LC2676). Gels were transferred to either nitrocellulose membrane (926-31090, LI-COR, Lincoln, NE, USA) or low-fluorescence polyvinylidene fluoride (PVDF) membrane (IPFL07810, LI-COR, Lincoln, NE, USA), followed by blocking with Odyssey blocking buffer (927-500000, LI-COR, Lincoln, NE, USA) or 5% milk in Tris buffer saline with 0.1% Tween 20 and staining with antibodies against GAA (ab137068, Abcam, Cambridge, MA, USA), anti-LC3B (L7543, Sigma-Aldrich, St. Louis, MO, USA), or anti-GAPDH (ab9484, Abcam, Cambridge, MA, USA) and the appropriate secondary (926-32213 or 925-68070, LI-COR, Lincoln, NE, USA). Blots were imaged with a LI-COR Odyssey CX. For Figure 1D only, the blots were imaged by the chemiluminescent method. Briefly, membranes were incubated with antibodies against GAA (ab113021, Abcam, Cambridge, MA, USA) and detected with anti-rabbit or anti-human horseradish peroxidase (HRP)-conjugated secondary antibodies (W4011 and W4031, Promega, Madison, WI, USA). Blots were developed with ECL (32106, Thermo Fisher, Waltham, MA, USA) and imaged on X-ray film (1651454, Carestream, Rochester, NY, USA).

**GAA enzymatic assay**

Cell lysates were assayed for GAA activity with the fluorogenic substrate 4-methylumbelliferyl-alpha-D-glucopyranoside. 4-Methylumbelliferone was used as a standard. Purified protein GAA activity used a commercial fluorescence assay kit (K187, BioVision, Milpitas, CA, USA). GAA activity was calculated as nanomoles of 4-methylumbelliferyl-alpha-D-glucopyranoside hydrolyzed per hour per nanomole of protein.

**Statistical analyses**

All statistical tests and analyses were performed in GraphPad Prism, version 7 or 8. For comparisons between anti-CD63:GAA and GAA in Figures 3 and 4, two-way ANOVAs with treatment and muscle tissue were performed, with multiple comparisons between treatments performed with a Tukey correction. Statistical significance was set at p < 0.05. To compare groups within the quadriceps in Figures 2 and 5, one-way ANOVAs were performed with post hoc Dunnett’s multiple comparison tests performed versus controls (untreated and wild-type mice) to determine significance. For grip strength and Rotarod test data, statistical significance was determined by two-way ANOVA (treatment and time factors) with Dunnett’s and Tukey’s multiple comparison tests. For ex vivo peak tetanic force analysis for Figure 6C, a one-way ANOVA with a post hoc Tukey’s test was used to compare treatment groups.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2021.08.020.

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**AUTHOR CONTRIBUTIONS**

A.D.B., X.Z., C.J.S., A.J.M., A.N.E., and K.D.C. designed the studies. A.D.B., P.C., N.A.A., and L.M. performed the mouse experiments. A. Mehra, S.M.-T., and C.A.K. designed and produced the transgenic mouse models. P.B. and N. Gale phenotyped the mouse models. S.B., Y.T., and A. Mujica designed and produced the transgenic AAVs. S.B., Y.T., and A. Mujica designed and produced the transgenic mouse models. P.B. and N. Gale phenotyped the mouse models.

**DECLARATION OF INTERESTS**

All authors were employees of Regeneron Pharmaceuticals, Inc., while engaged in the study and may hold stock and/or stock options in the company. A.D.B. and K.D.C. have patent applications for internalizing enzymes and uses thereof.
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