Exploiting the diphtheria toxin internalization receptor enhances delivery of proteins to lysosomes for enzyme replacement therapy

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Enzyme replacement therapy, in which a functional copy of an enzyme is injected either systemically or directly into the brain of affected individuals, has proven to be an effective strategy for treating certain lysosomal storage diseases. The inefficient uptake of recombinant enzymes via the mannose-6-phosphate receptor, however, prohibits the broad utility of replacement therapy. Here, to improve the efficiency and efficacy of lysosomal enzyme uptake, we exploited the strategy used by diphtheria toxin to enter into the endolysosomal network of cells by creating a chimera between the receptor-binding fragment of diphtheria toxin and the lysosomal hydrolase TPP1.

INTRODUCTION

Lysosomal storage diseases (LSDs) are a group of more than 70 inherited childhood diseases characterized by an accumulation of cellular metabolites arising from deficiencies in a specific protein, typically a lysosomal hydrolase. Although each individual disease is considered rare, LSDs have a combined incidence of between 1/5000 and 1/8000 live births, and together, they account for a substantial proportion of the neurodegenerative diseases in children (1). The particular age of onset for a given LSD varies depending on the affected protein and the percentage of enzymatic activity still present; however, in most cases, symptoms manifest early in life and progress insidiously, affecting multiple tissues and organs (2). In all but the mildest of cases, disease progression results in severe physical disability, possible intellectual disability, and a shortened life expectancy, with death occurring in late childhood or early adolescence.

As they are monogenic diseases, reintroducing a functional form of the defective enzyme into lysosomes is in principle a viable strategy for treating LSDs. Enzyme replacement therapy (ERT) is now approved for the treatment of seven LSDs, and clinical trials are ongoing for five others (3). However, delivering curative doses of recombinant lysosomal enzymes into lysosomes remains a major challenge in practice. ERT typically takes advantage of a specific N-glycan post-translational modification, mannose-6-phosphorylation (M6P), which controls trafficking of endogenous lysosomal enzymes, as well as exogenous uptake of lysosomal enzymes from circulation by cells having the cation-independent M6P receptor (CIMPR) (4). Hence, a combination of factors including (i) the abundance of the M6P receptor in the liver, (ii) poor levels of CIMPR expression in several key target tissue types such as bone and skeletal muscle, (iii) incomplete and unpredictable M6P labeling of recombinant enzymes, and (iv) the highly variable affinity of recombinant lysosomal enzymes for CIMPR (viz., \( K_d \)'s (dissociation constants) ranging from low to mid micromolar (5, 6)) all contribute to diminishing the overall effectiveness of therapies using CIMPR for cell entry (3).

To improve the delivery of therapeutic lysosomal enzymes, we drew inspiration from bacterial toxins, which, as part of their mechanism, hijack specific host cell–surface receptors to gain entry into the endolysosomal pathway. While we and others have explored exploiting this pathway to deliver cargo into the cytosol (7, 8), here we asked whether this same approach could be used to enhance the delivery of lysosomal enzymes into lysosomes. We choose the diphtheria toxin (DT)–diphtheria toxin receptor (DTR) system owing to the ubiquitous nature of the DTR, in particular its high expression levels on neurons.

*Corynebacterium diphtheriae* secretes DT exotoxin, which is spread to distant organs by the circulatory system, where it affects the lungs, heart, liver, kidneys, and the nervous system (9). It is estimated that 75% of individuals with acute disease also develop some form of peripheral or cranial neuropathy. This multiorgan targeting results from the fact that the DTR, heparin-binding EGF (epidermal growth factor)–like growth factor (HBEGF), is ubiquitously expressed. The extent to which DT specifically targets difficult-to-access tissues such as muscle and bone, however, is not currently known.

DT is a three-domain protein that consists of an N-terminal ADP (adenosine diphosphate)–riboosyl transferase enzyme (DT\(_C\)), a central translocation domain (DT\(_T\)), and a C-terminal receptor–binding domain (DT\(_R\)). The latter is responsible for both binding cell surface HBEGF with high affinity (viz., \( K_d = 27 \text{ nM} \) (10)) and triggering endocytosis into early endosomes (Fig. 1A). Within endosomes, DT\(_T\) forms membrane-spanning pores that serve as conduits for DT\(_C\) to enter the cytosol where it inactivates the host protein synthesis machinery. The remaining portions of the toxin remain in the endosomes and continue to lysosomes where they are...
We showed that the feasibility of delivering therapeutic enzymes into lysosomes through the DT-HBEGF internalization pathway. We hypothesized that the receptor-binding domain, DT R , with the goal of demonstrating its ability to fold and bind cell surface HBEGF. Before treating cells with a fixed dose of wild-type DT that completely inhibits protein synthesis, cells were incubated with a range of concentrations of LTM or a full-length, nontoxic mutant of DT (DTK51E/E148K ). LTM-mediated inhibition of wild-type DT-mediated toxicity was equivalent to nontoxic DT (Fig. 1B), demonstrating that the receptor-binding fragment can be isolated from the holotoxin without affecting its ability to fold and bind cell surface HBEGF. Next, we evaluated whether LTM had a positional bias (i.e., was able to bind HBEGF with a fusion partner when positioned at either terminus). To this end, we generated N- and C-terminal fusions of LTM to the model fluorescent protein mCherry (i.e., mCherry-LTM and LTM-mCherry). To determine binding of each chimera to HBEGF, we quantified the ability of each chimera to compete with wild-type DT on cells in the intoxication assay. Both constructs competed with wild-type DT to the same extent as LTM alone and DTK51E/E148K (Fig. 1C), demonstrating that LTM is versatile and autonomously folds in different contexts.

To evaluate intracellular trafficking, HeLa cells were treated with either LTM-mCherry or mCherry-LTM and then fixed and stained 4 hours later with an antibody against the lysosomal marker LAMP1. In both cases, we observed significant uptake of the fusion protein (Fig. 1, D and E). We calculated Manders’ coefficients (M2) to quantify the extent to which signal in the red channel (LTM-mCherry and LTM-TPP1) was localized with signal in the green channel (LAMP1). The fraction of red/green co-occurrence was calculated to be 0.61 for mCherry-LTM and 0.52 for LTM-mCherry, indicating trafficking to the lysosomal compartments of the cells and no significant difference (P = 0.196) between the two orientations of chimera (Fig. 1F). Together, these results confirm that the LTM is capable of binding HBEGF and trafficking associated cargo into cells and that the LTM can function in this manner at either terminus of a fusion construct.

**LTM-TPP1 retains TPP1 enzymatic activity and HBEGF binding**

With minimal positional bias observed in the mCherry fusion proteins, we next screened LTM fusions to TPP1 to identify a design that maximizes expression, stability, activity, and, ultimately, delivery. TPP1 is a 60-kDa lysosomal serine peptidase encoded by the CLN2 gene, implicated in neuronal ceroid lipofuscinosis type 2 or Batten disease. Loss of function results in the accumulation of lipofuscin, a proteinaceous, autofluorescent storage material. The low-pH environment of the lysosome triggers autoproteolytic activation of TPP1 and release of a 20-kDa propeptide that occludes its active site. From a design perspective, we favored an orientation in which the LTM was N terminal to TPP1, as autoprocessing of TPP1 would result in the release of the upstream LTM-TPP1 propeptide, liberating active, mature TPP1 enzyme in the lysosome (Fig. 2A). Given the need for mammalian expression of lysosomal enzymes, we generated synthetic genetic fusions of the LTM to TPP1, in which we converted the codons from bacterially derived DT into the corresponding mammalian codons. Human embryonic kidney (HEK) 293F suspension cells stably expressing recombinant TPP1 (rTPP1) and TPP1 with an N-terminal LTM fusion (LTM-TPP1) were generated using the piggyBac transposon system. A C-terminal

**RESULTS**

**The DTR-binding fragment is an autonomous LTM**

To evaluate whether the DTR-binding fragment could function autonomously to traffic cargo into lysosomes, we first asked whether the isolated 17-kDa DT R fragment could be expressed independently from DT holotoxin and retain its affinity for HBEGF. We cloned, expressed, and purified the receptor-binding fragment and evaluated its ability to compete with full-length DT for the DTR, HBEGF. Before treating cells with a fixed dose of wild-type DT that completely inhibits protein synthesis, cells were incubated with a range of concentrations of LTM or a full-length, nontoxic mutant of DT (DTK51E/E148K). LTM-mediated inhibition of wild-type DT-mediated toxicity was equivalent to nontoxic DT (Fig. 1B), demonstrating that the receptor-binding fragment can be isolated from the holotoxin without affecting its ability to fold and bind cell surface HBEGF. Next, we evaluated whether LTM had a positional bias (i.e., was able to bind HBEGF with a fusion partner when positioned at either terminus). To this end, we generated N- and C-terminal fusions of LTM to the model fluorescent protein mCherry (i.e., mCherry-LTM and LTM-mCherry). To determine binding of each chimera to HBEGF, we quantified the ability of each chimera to compete with wild-type DT on cells in the intoxication assay. Both constructs competed with wild-type DT to the same extent as LTM alone and DTK51E/E148K (Fig. 1C), demonstrating that LTM is versatile and autonomously folds in different contexts.

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construct (TPP1-LTM) was also produced; however, expression of this chimera was poor in comparison with rTPP1 and LTM-TPP1 (~0.4 mg/liter, cf. 10 to 15 mg/liter).

The activity of rTPP1 and LTM-TPP1 against the tripeptide substrate Ala-Ala-Phe-AMC (AAF-AMC) was assessed to determine any effects of the LTM on TPP1 activity. The enzyme activities of rTPP1 and LTM-TPP1 were determined to be equivalent, as evidenced through measurements of their catalytic efficiency (Fig. 2B), demonstrating that there is no inference by LTM on the peptidase activity of TPP1. Maturation of LTM-TPP1 through autocatalytic cleavage of the N-terminal propeptide was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 2C). Complete processing of the zymogen at pH 3.5 and 37°C occurred between 5 and 10 min, which is consistent with what has been observed for the native recombinant enzyme (15).

The ability of LTM-TPP1 to compete with DT for binding to extracellular HBEGF was first assessed with the protein synthesis competition assay. Similar to LTM, mCherry-LTM, and LTM-mCherry, LTM-TPP1 prevents protein synthesis inhibition by 10 pM DT with an IC₅₀ (median inhibitory concentration) of 17.2 nM (Fig. 2D). As expected, rTPP1 alone was unable to inhibit DT-mediated entry and cytotoxicity. To further characterize this interaction, we measured the interaction between LTM and LTM-TPP1 and recombinant HBEGF using surface plasmon resonance (SPR) binding analysis (Fig. 2E). By SPR, LTM and LTM-TPP1 were calculated to have apparent Kₛₐ's of 13.3 and 19.1 nM, respectively, consistent with what has been observed for the native recombiant enzyme (15).

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CRISPR-Cas9–mediated knockout of CLN2 in HeLa Kyoto cells

To study uptake of chimeric fusion proteins in cell culture, we generated a cell line deficient in TPP1 activity. A CRISPR RNA (crRNA) was designed to target the signal peptide region of TPP1 in exon 2 of CLN2. Human HeLa Kyoto cells were reverse transfected with a Cas9 ribonucleoprotein complex and then seeded at low density into a 10-cm dish. Single cells were expanded to colonies, which were picked and screened for TPP1 activity. A single clone deficient in TPP1 activity was isolated and expanded, which was determined to have ~4% TPP1 activity relative to wild-type HeLa Kyoto cells plated at the same density (Fig. 3A). The small residual activity observed is likely the result of another cellular enzyme processing the AAF–AMC (7-amido-4-methlycoumarin) substrate used in this assay, as there is no apparent TPP1 protein being produced (Fig. 3B). Sanger sequencing of the individual alleles confirmed complete disruption of the CLN2 gene (fig. S1). In total, three unique mutations were identified within exon 2 of CLN2: a single base insertion resulting in a frameshift mutation and two deletions of 24 and 33 base pairs (bp), respectively.

LTM-TPP1 uptake into cells is more efficient than free rTPP1

Next, we compared the delivery and activation of rTPP1 and LTM-TPP1 into lysosomes by treating TPP1−/− cells with a fixed concentration of the enzymes (100 nM) and by analyzing entry and processing by Western blot (Fig. 3C). In both cases, most enzymes were present in the mature form, indicating successful delivery to the lysosome; however, the uptake of LTM-TPP1 greatly exceeded the uptake of rTPP1. As both rTPP1 and LTM-TPP1 receive the same M6P post-translational modifications promoting their uptake by CIMPR,
differences in their respective uptake should be directly attributable to uptake by HBEGF. To quantify the difference in uptake and lysosomal delivery, cells were treated overnight with varying amounts of each enzyme, washed, lysed, and assayed for TPP1 activity. The activity assays were performed without a preactivation step, so signal represents protein that has been activated in the lysosome. For both constructs, we observed a dose-dependent increase in delivery of TPP1 to the lysosome (Fig. 3D). Delivery of LTM-TPP1 was significantly enhanced compared with TPP1 alone at all doses, further demonstrating that uptake by HBEGF is more efficient than that by CIMPR alone. TPP1 activity in cells treated with LTM-TPP1 was consistently ~10× greater than that of cells treated with rTPP1, with the relative difference increasing at the highest concentrations tested. This may speak to differences in abundance, replenishment, and/or recycling of HBEGF versus CIMPR, in addition to differences in receptor-ligand affinity. Uptake of LTM-TPP1 and rTPP1 into several other cell types yielded similar results (fig. S2). To assess the lifetime of the delivered enzyme, cells were treated with LTM-TPP1 (50 nM) and incubated overnight. Cells were washed and incubated with fresh media, and TPP1 activity was assayed over the course of several days. Cells treated with LTM-TPP1 still retained measurable TPP1 activity at 1 week after treatment (Fig. 3E).

While the DT competition experiment demonstrated that HBEGF is involved in the uptake of LTM-TPP1 but not rTPP1 (Fig. 2D), it does not account for the contribution of CIMPR to uptake. Endoglycosidase H (EndoH) cleaves between the core N-acetylglucosamine residues of high-mannose N-linked glycans, leaving behind only the asparagine-linked N-acetylglucosamine moiety. Both rTPP1 and LTM-TPP1 were treated with EndoH to remove any M6P moieties, and delivery into HeLa Kyoto TPP1−/− cells was monitored by TPP1 activity (means ± SD; n = 4). (E) TPP1 activity present in HeLa Kyoto TPP1−/− cells following a single treatment with 50 nM LTM-TPP1 (means ± SD; n = 3).

Fig. 3. Intracellular uptake of LTM-TPP1 into CLN2 knockout cells. (A) CLN2 knockout cells exhibit ~4% TPP1 activity relative to wild-type HeLa Kyoto cells (means ± SD; n = 3). (B) Western blotting against TPP1 reveals no detectable protein in the knockout cells. (C) (Left) In vitro maturation of pro-rTPP1 and LTM-TPP1 (16 ng) was analyzed by Western blot. (Right) TPP1 present in wild-type (WT) and TPP1−/− cells, and TPP1−/− cells treated with 100 nM rTPP1 and LTM-TPP1. (D) Uptake of rTPP1 and LTM-TPP1 into HeLa Kyoto TPP1−/− cells was monitored by TPP1 activity (means ± SD; n = 4). (E) TPP1 activity present in HeLa Kyoto TPP1−/− cells following a single treatment with 50 nM LTM-TPP1 (means ± SD; n = 3).

Fig. 4. Uptake of rTPP1 and LTM-TPP1 into HeLa Kyoto TPP1−/− cells following EndoH treatment to remove N-glycan labeling (means ± SD; n = 3).

by which LTM-TPP1 is taken up into cells, uptake via CIMPR still occurs. The fact that CIMPR uptake is still possible in the LTM-TPP1 fusion means that the fusion is targeted to two receptors simultaneously, increasing its total uptake and, potentially, its biodistribution.

Intracerebroventricular injection of LTM-TPP1 is taken up more efficiently into murine brain tissue than rTPP1 alone.

Uptake of LTM-TPP1 via the combination of HBEGF and CIMPR was shown to be 3x to 20x more efficient than CIMPR alone in cellulo (fig. S2). To interrogate this effect in vivo, TPP1-deficient mice (TPP1tm1pLob or TPP1−/−) were obtained as a gift from P. Lobel at Rutgers University. Targeted disruption of the CLN2 gene was achieved by insertion of a neo cassette into intron 11 in combination with a point mutation (R446H), rendering these mice TPP1 null by both Western blot and enzyme activity assay (16). Prior studies have demonstrated that direct administration of rTPP1
into the cerebrospinal fluid (CSF) via intracerebroventricular or intrathecal injection results in amelioration of disease phenotype (17) and even extension of life span in the disease mouse (18). To compare the uptake of LTM-TPP1 and rTPP1 in vivo, the enzymes were injected into the left ventricle of 6-week-old TPP1−/− mice. Mice were euthanized 24 hours after injection, and brain homogenates of wild-type littermates, untreated, and treated mice were assayed for TPP1 activity (Fig. 5A). Assays were performed without preactivation, and therefore, the results report on enzyme that has been taken up into cells, trafficked to the lysosome, and processed to the mature form.

While both enzymes resulted in a dose-dependent increase in TPP1 activity, low (5 μg) and high (25 μg) doses of rTPP1 resulted in only modest increases of activity, representing ~6 and ~26% of the wild-type levels of activity, respectively (Fig. 5B). At the same doses, LTM-TPP1 restored ~31 and ~103% of the wild-type activity.

To assess the lifetime of enzyme in the brain, mice were injected intracerebroventricularly with 25 μg of LTM-TPP1 and euthanized either 1 or 2 weeks postinjection. Remarkably, at 1 week postinjection, ~68% of TPP1 activity was retained (compared with 1 day postinjection), and after 2 weeks, activity was reduced to ~31% (Fig. 5C).

**DISCUSSION**

ERT is a lifesaving therapy that is a principal method of treatment in non-neurological LSDs. Uptake of M6P-labeled enzymes by CIMPR is relatively ineffective due to variable receptor affinity (5, 6), heterogeneous expression of the receptor, and incomplete labeling of recombinantly produced enzymes (19). Despite its inefficiencies and high cost (~200,000 USD per patient per year) (20), it remains the standard of care for several LSDs, as alternative treatments (substrate reduction therapy, gene therapy, and hematopoietic stem cell transplantation) are not effective, not as well developed, or inherently riskier (21–25). Improving the efficiency and distribution of recombinant enzyme uptake may help address some of the current shortcomings in traditional ERT.

Several strategies have been used to increase the extent of M6P labeling on recombinantly produced lysosomal enzymes: engineering mammalian and yeast cell lines to produce more specific/uniform N-glycan modification (19, 26, 27), chemical or enzymatic modification of N-glycans posttranslationally (28), and covalent coupling of M6P (29). M6P-independent uptake of a lysosomal hydrolase by CIMPR has been demonstrated for both β-glucuronidase (28) and acid α-glucosidase (30, 31). In the latter work, a peptide tag (GILT) targeting insulin-like growth factor II receptor (IGF2R) was fused to recombinant alpha glucosidase, which enabled receptor-mediated entry into cells. CIMPR is a ~300-kDa, 15-domain membrane protein with 3 M6P-binding domains and 1 IGF2R domain. By targeting the IGF2R domain with a high-affinity (low nanomolar) peptide rather than the low-affinity M6P-binding domain, the authors were able to demonstrate a ~20-fold increase in the uptake of a GAA-peptide fusion protein in cell culture and a ~5-fold increase in the ability to clear built-up muscle glycogen in GAA-deficient mice.

In this study, we have demonstrated efficient uptake and lysosomal trafficking of a model lysosomal enzyme, TPP1, via a CIMPR-independent route, using the receptor-binding domain of a bacterial toxin. HBEGF is a member of the EGF family of growth factors, and DT is its only known ligand. Notably, it plays roles in cardiac development, wound healing, muscle contraction, and neurogenesis; however, it does not act as a receptor in any of these physiological processes (32). Intracellular intoxication by DT is the only known process in which HBEGF acts as a receptor, making it an excellent candidate receptor for ERT, as there is no natural ligand with which to compete. Upon binding, DT is internalized via clathrin-mediated endocytosis and then trafficked toward lysosomes for degradation (33, 34). Acidification of endosomal vesicles by vacuolar ATPases (adenosine triphosphatases) promotes insertion of DT into the endosomal membrane and subsequent translocation of the catalytic DTc domain into the cytosol. In the absence of an escape mechanism, the majority of internalized HBEGF should be trafficked to the lysosome, as we have demonstrated with our chimera (Figs. 2F and 3C). Uptake of LTM-TPP1 in vitro is robustly relative to rTPP1 (Fig. 3D and fig. S2), and TPP1 activity is sustained in the lysosome for a substantial length of time (Fig. 3E). We have also demonstrated that the increase in uptake efficiency that we observed in cell culture persists in vivo. TPP1 activity in the brains of CLN2-null mice was significantly greater in animals treated with intracerebroventricularly injected LTM-TPP1, as compared with those treated with TPP1 at two different doses (Fig. 5B), and, remarkably, this activity persists with an apparent half-life of ~8 days (Fig. 5C).

An important consideration for further development of the LTM platform for clinical development is the potential immunogenicity of using a bacterial fragment in this context. Previously, we demonstrated that the receptor-binding fragment of DT could be replaced with a human scFv (single-chain fragment variable) targeting HBEGF (8). With our demonstration of the potential for targeting HBEGF for LSDs, future efforts will focus on increasing the affinity and specificity of these first-generation humanized LTMs to develop high-affinity chimeras with greatly reduced immunogenicity for further development.
While the ability of LTM-TPP1 to affect disease progression has yet to be determined, recent positive clinical trial results (35) and the subsequent approval of rTPP1 (cerliponase alfa) for treatment of neuronal ceroid lipofuscinosis 2 (NCL2) provide support for this approach. In that clinical trial, 300 mg of rTPP1 was administered by biweekly intracerebroventricular injection to 24 affected children, and this was able to prevent disease progression. While this dose is of the same order of magnitude as other approved ERTs (<1 to 40 mg/kg) (36, 37), it represents a substantial dose, especially considering that it was delivered to a single organ. Improving the efficiency of uptake by targeting an additional receptor as we have done here, is expected to greatly decrease the dose required to improve symptoms, while at the same time decreasing costs and the chances of dose-dependent side effects.

**MATERIALS AND METHODS**

**Protein expression and purification**

DTK51E/E148K, LTM, LTM-mCherry, mCherry-LTM, and HBEGF constructs were cloned using the In-Fusion HD cloning kit (Clontech) into the Champion pET SUMO expression system (Invitrogen). Recombinant proteins were expressed as 6His-SUMO fusion proteins in *Escherichia coli* BL21(DE3)pLysS cells. Cultures were grown at 37°C until an OD560 (optical density at 600 nm) of 0.5, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 hours at 25°C. Cell pellets harvested by centrifugation were resuspended in lysis buffer [20 mM tris (pH 8.0), 160 mM NaCl, 10 mM imidazole, lysozyme, benzonase, and protease inhibitor cocktail] and lysed by three passages through an EmulsiFlex C3 microfluidizer (Avestin). Following clarification by centrifugation at 18,000 g/l for 20 min and syringe filtration (0.2 μm), soluble lysate was loaded over a 5-ml His-trap FF column (GE Healthcare) using an AKTA FPLC. Bound protein was washed and eluted over an imidazole gradient (20 to 150 mM). Fractions were assessed for purity by SDS-PAGE, pooled, concentrated, and frozen on dry ice in 25% glycerol for storage at −80°C.

TPP1 cDNA was obtained from the SPARC BioCentre (The Hospital for Sick Children) and cloned into the piggyBac plasmid pB-T-PAF (J.M.R., University of Toronto) using Not I and Asc I restriction sites to generate two expression constructs (pB-T-PAF-ProteinA-TEV-LTM-TPP1 and pB-T-PAF-ProteinA-TEV-TPP1). Stably transfected expression cell lines (HEK293F) were then generated using the piggyBac transposon system, as described (14). Protein expression was induced with doxycycline, and secreted fluorescent TPP1 and mCherry-LTM were purified after 14 hours (380 nm excitation/460 nm emission) arising from the release of AMC [50 mM NaOAc (pH 3.5) and 100 mM NaCl] and substrate (200 μM AAF-AMC) were then added to a final volume of 100 μl of activation buffer [50 mM NaOAc (pH 3.5) and 100 mM NaCl] for 1 hour at 37°C. Assay buffer [50 mM NaOAc (pH 5.0) and 100 mM NaCl] and substrate (200 μM AAF-AMC) were then added to a final volume of 100 μl. Fluorescence (380 nm excitation/460 nm emission) arising from the release of AMC was monitored in real time using a SpectraMax M5e (Molecular Devices). TPP1 activity in cellulo was measured similarly, without the activation step. Cells in a 96-well plate were incubated with 25 μl of 0.5% Triton X-100 in PBS, which was then transferred to a black 96-well plate containing 75 μl of assay buffer with substrate in each well.

**In vitro TPP1 activity assay**

TPP1 protease activity was measured using the synthetic substrate AAF-AMC using a protocol adapted from Vines and Warburton (38). Briefly, enzyme was preactivated in 25 μl of activation buffer [50 mM NaOAc (pH 3.5) and 100 mM NaCl] for 1 hour at 37°C. Assay buffer [50 mM NaOAc (pH 5.0) and 100 mM NaCl] and substrate (200 μM AAF-AMC) were then added to a final volume of 100 μl. Fluorescence (380 nm excitation/460 nm emission) arising from the release of AMC was monitored in real time using a SpectraMax M5e (Molecular Devices). TPP1 activity in cellulo was measured similarly, without the activation step. Cells in a 96-well plate were incubated with 25 μl of 0.5% Triton X-100 in PBS, which was then transferred to a black 96-well plate containing 75 μl of assay buffer with substrate in each well.

**Generation of CLN2−/− cell line by CRISPR-Cas9**

crRNA targeting the signal peptide sequence in exon 2 of CLN2 was designed using the Integrated DNA Technologies (www.idtdna.com) design tool. The gRNA:Cas9 ribonucleoprotein complex was assembled...
according to the manufacturer’s protocol (Integrated DNA Technologies) and reverse transected using Lipofectamine RNAiMAX (Thermo Fisher Scientific) into HeLa Kyoto cells (40,000 cells in a 96-well plate). Following 48 hours of incubation, 5000 cells were seeded into a 10-cm dish. Clonal colonies were picked after 14 days and transferred to a 96-well plate. Clones were screened for successful CLN2 knockout by assaying TPP1 activity and confirmed by Sanger sequencing and Western blot against TPP1 antibody (Abcam, ab54385).

Autoactivation
The pro-form of TPP1 was matured in vitro to the active form in 50 mM NaOAc (pH 3.5) and 100 mM NaCl for 1 to 30 min at 37°C. The autoactivation reaction was halted by the addition of 2× Laemmli SDS sample buffer containing 10% 2-mercaptoethanol and boiled for 5 min. Pro and mature TPP1 were separated by SDS-PAGE and imaged on a ChemiDoc gel imaging system (Bio-Rad).

Western blot
Proteins or cellular lysate were separated by 4 to 20% gradient SDS-PAGE before being transferred to a nitrocellulose membrane using the iBlot (Invitrogen) dry transfer system. Membranes were then blocked for 1 h with a 5% milk–Tris-buffered saline (TBS) solution and incubated overnight at room temperature with a 1:100 dilution of mouse monoclonal antibody against TPP1 (Abcam, ab54685) in 5% milk–TBS. Membranes were washed 3 × 5 min with 0.1% Tween 20 (Sigma-Aldrich) in TBS before a 1-hour incubation with a 1:5000 dilution of sheep anti-mouse IgG horseradish peroxidase secondary antibody (GE Healthcare) in 5% milk–TBS. The membranes were washed 3 × 5 min with 0.1% Tween 20 (Sigma-Aldrich) in TBS before a 1-hour incubation with a 1:5000 dilution of sheep anti-mouse IgG horseradish peroxidase secondary antibody (GE Healthcare) in 5% milk–TBS. Chemiluminescent signal was developed with Clarity Western ECL substrate (Bio-Rad) and visualized on a ChemiDoc gel imaging system (Bio-Rad).

Treatment with EndoH
rTTP1 and LTM-TTP1 were treated with EndoH (New England Bio-labs) to remove N-glycan modifications. Enzymes were incubated at 1 mg/ml with 2500 U of EndoH for 48 hours at room temperature in 20 mM tris (pH 8.0) and 150 mM NaCl in a total reaction volume of 20 μl. Cleavage of N-glycans was assessed by SDS-PAGE, and concentrations were normalized to native enzyme-specific activities.

Animals
Cryopreserved TPP1+/− embryos were obtained from P. Lobel at Rutgers University and rederived in a C57/BL6 background at The Centre for Phenogenomics. Animal care and all procedures were approved by The Centre for Phenogenomics Animal Care Committee and are in compliance with the CCAC (Canadian Council on Animal Care) guidelines and the OMAFRA (Ontario Ministry of Agriculture, Food, and Rural Affairs) Animals for Research Act.

Intracerebroventricular injection
TPP1+/− mice (60 days old) were anesthetized with isoflurane (inhaled) and injected subcutaneously with sterile saline (1 ml) and meloxicam (2 mg/kg). Mice were secured to a stereotaxic system, a small area of the head was shaved, and a single incision was made to expose the skull. A high-speed burr was used to drill a hole at stereotaxic coordinates: anteroposterior (A/P), −1.0 mm; mediolateral (M/L), −0.3 mm; and dorsoventral (D/V), −3.0 mm relative to the bregma, and a 33-gauge needle attached to a 10-μl Hamilton syringe was used to perform the intracerebroventricular injection into the left ventricle. Animals received either 1 or 5 μl of enzyme (5 μg/μl), injected at a constant rate. Isoflurane-anesthetized animals were euthanized by transcardial perfusion with PBS. Brains were harvested and frozen immediately, then thawed and homogenized in lysis buffer [500 mM NaCl, 0.5% Triton X-100, 0.1% SDS, and 50 mM Tris (pH 8.0)] using 5-mm stainless steel beads in TissueLyser II (Qiagen). In vitro TPP1 assay was performed, as described, minus the activation step.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/50/eabb0385/DC1

REFERENCES AND NOTES
1. F. M. Platt, B. Boland, A. C. van der Spoel, Lysosomal storage disorders: The cellular impact of lysosomal dysfunction. J. Cell Biol. 199, 723–734 (2012).
2. G. Parenti, G. Andria, B. Ballabio, Lysosomal storage diseases: From pathophysiology to therapy. Annu. Rev. Med. 66, 471–486 (2015).
3. R. J. Desnick, E. H. Schuchman, Enzyme replacement therapy for lysosomal diseases: Lessons from 20 years of experience and remaining challenges. Annu. Rev. Genomics Hum. Genet. 13, 307–335 (2012).
4. W. S. Sly, H. D. Fischer, The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. J. Cell Biochem. 18, 67–85 (1982).
5. P. Y. Yong, S. Kornfeld, Ligand interactions of the cation-dependent mannose 6-phosphate receptor. Comparison with the cation-independent mannose 6-phosphate receptor. J. Biol. Chem. 264, 7970–7975 (1989).
6. R. N. Bohnsack, X. Song, L. J. Olson, M. Kudo, R. R. Gotschall, W. M. Canfield, R. D. Cummings, D. F. Smith, N. M. Dahms, Cation-independent mannose 6-phosphate receptor: A composite of distinct phosphomannosyl binding sites. J. Biol. Chem. 284, 35215–35226 (2009).
7. A. Auger, M. Park, F. Nitschke, L. M. Minnassian, G. L. Beilhartz, B. A. Minassian, R. A. Melnyk, Efficient delivery of structurally diverse protein cargo into mammalian cells by a bacterial enzyme, Mol. Pharm. 12, 2962–2971 (2015).
8. M. Park, X. Xu, W. Min, S. N. Sugiman-Marangos, G. L. Beilhartz, J. J. Adams, S. S. Sidhu, E. Grunebaum, R. A. Melnyk, Intracellular delivery of human purine nucleoside phosphorylase by engineered diphtheria toxin rescues function in target cells, Mol. Pharm. 15, 5217–5226 (2018).
9. T. L. Hadfield, P. McEvoy, Y. Polotsky, V. A. Timmerling, A. A. Yakovlev, The pathology of diphtheria. J Infect Dis 181 (Suppl. 1), S116–S120 (2000).
10. J. S. Brooke, J. H. Cha, L. Eidel, Diphtheria toxin/receptor interaction: Association, dissociation, and effect of pH. Biochem. Biophys. Res. Commun. 248, 297–302 (1998).
11. R. E. Morris, A. S. Gerstein, P. F. Bonventre, C. B. Saelinger, Receptor-mediated entry of diphtheria toxin into kidney Vero (Vero) cells: Electron microscopic evaluation. Infect. Immun. 50, 721–727 (1985).
12. E. Lemiche, M. Bomsel, G. Devillers, J. van der Spek, J. R. Murphy, E. V. Lukianov, S. Olnes, P. Boquet, Membrane translocation of diphtheria toxin fragment A exploits early to late endosome trafficking machinery. Mol. Microbiol. 23, 445–457 (1997).
13. M. Halla, H. H. Goebel, The neuronal ceroid-lipофuscinoses: A historical introduction. Biochim. Biophys. Acta 1832, 1795–1800 (2013).
14. Z. Li, P. Michael, D. Zhou, A. Nagy, J. M. Rini, Simple piggyBac transposon-based mammalian cell expression system for inducible protein production. Proc. Natl. Acad. Sci. U.S.A. 110, 5004–5009 (2013).
15. J. G. Ungar, I. Sohar, K. Das, A. M. Stock, P. Lobel, Crystal structure and autoactivation pathway of the precursor form of human tripetidyl-peptidase 1, the enzyme deficient in late infantile ceroid lipofuscinosis. J. Biol. Chem. 284, 3985–3997 (2009).
16. D. E. Sleat, J. A. Wiseman, M. El-Banna, K. H. Kim, Q. Mao, S. Price, S. L. Macauley, R. L. Sidman, M. M. Shen, Q. Zhao, M. A. Passini, B. L. Davidson, G. R. Steward, P. Lobel, A mouse model of classical late-infantile neuronal ceroid lipofuscinosis based on targeted disruption of the CLN2 gene results in a loss of tripetidyl-peptidase I activity and progressive neurodegeneration. J. Neurosci. 24, 9117–9126 (2004).
17. M. Chang, J. D. Cooper, D. E. Sleat, S. H. Cheng, J. C. Dodge, M. A. Passini, P. Lobel, B. L. Davidson, Intraventricular enzyme replacement improves disease phenotypes in a mouse model of late-infantile neuronal ceroid lipofuscinosis. Mol. Ther. 16, 649–656 (2008).
18. S. Xu, L. Wang, M. El-Banna, I. Sohar, D. E. Sleat, P. Lobel, Large-volume intrathecal enzyme delivery increases survival of a mouse model of late infantile neuronal ceroid lipofuscinosis. Mol. Ther. 19, 1842–1848 (2011).
Sugiman-Marangos et al., Sci. Adv. 2020; 6 : eabb0385 11 December 2020

19. D. B. Oh, Glyco-engineering strategies for the development of therapeutics with improved efficacy for the treatment of lysosomal storage diseases. BMB Rep. 48, 438–444 (2015).

20. J. E. Wraith, Limitations of enzyme replacement therapy: Current and future. J. Inherit. Metab. Dis. 29, 442–447 (2006).

21. V. Valayannopoulos, Enzyme replacement therapy and substrate reduction therapy in lysosomal storage disorders with neurological expression. Handb. Clin. Neurol. 113, 1851–1857 (2013).

22. A. Biffi, Gene therapy for lysosomal storage disorders: A good start. Hum. Mol. Genet. 25, R65–R75 (2016).

23. C. E. Dunbar, K. A. High, J. K. Joung, D. B. Kohn, K. Ozawa, M. Sadelain, Gene therapy comes of age. Science 359, eaan4672 (2018).

24. M. Aldenhoven, R. F. Wynn, P. J. Orchard, A. O’Meara, P. Veys, A. Fischer, V. Valayannopoulos, B. Neven, A. Rovelli, V. K. Prasad, J. Tolar, H. Alleveld, S. A. Jones, R. Parini, M. Renard, V. Bordron, N. M. Wulffraat, T. J. de Koning, E. G. Shapiro, J. Kurtzberg, J. J. Boelens, Long-term outcome of Hurler syndrome patients after hematopoietic cell transplantation: An international multicenter study. Blood 125, 2164–2172 (2015).

25. J. J. Boelens, V. K. Prasad, J. Tolar, R. F. Wynn, C. Peters, Current international perspectives on hematopoietic stem cell transplantation for inherited metabolic disorders. Pediatr. Clin. North Am. 57, 123–145 (2010).

26. T. Tsukimura, I. Kawashima, T. Togawa, T. Kodama, T. Suzuki, T. Watanabe, Y. Chiba, Y. Jimoto, T. Fukushima, T. Kanekura, H. Sakuraba, Efficient uptake of recombinant α-galactosidase A produced with a gene-manipulated yeast by Fabry mice kidneys. Mol. Med. 18, 76–82 (2012).

27. L. Liu, W. S. Lee, B. Doray, S. Komfeld, Engineering of GlcNAc-1-phosphotransferase for production of highly phosphorylated lysosomal enzymes for enzyme replacement therapy. Mol. Ther. Methods Clin. Dev. 5, 59–65 (2017).

28. B. Friedman, K. Vaddi, C. Preston, E. Mahon, J. R. Cataldo, J. M. McPherson, A comparison of the pharmacological properties of carbohydrate remodeled recombinant and placental-derived β-glucocerebrosidase: implications for clinical efficacy in treatment of Gaucher disease. Blood 93, 2808–2816 (1999).

29. J. Y. Kang, K. K. Shin, H. K. Kim, J. K. Min, E. S. Ji, J. Y. Kim, O. Kwon, D. B. Oh, Lysosomal targeting enhancement by conjugation of glycopeptides containing mannose-6-phosphate glycans derived from glyco-engineered yeast. Sci. Rep. 8, 8730 (2018).

30. G. Horning, S. Rasmussen, A. Permin, M. Bisgaard, Investigations on the influence of helminth parasites on vaccination of chickens against Newcastle disease virus under village conditions. Trop. Anim. Health Prod. 35, 415–424 (2003).

31. J. A. Maga, J. Zhou, R. Kambampati, S. Peng, X. Wang, R. N. Bohnsack, A. Thomm, S. Golata, P. Tom, N. M. Dahms, B. J. Byrne, J. H. LeBowitz, Glycosylation-independent lysosomal targeting of acid α-glucosidase enhances muscle glycolen clearance in Pompe mice. J. Biol. Chem. 288, 1428–1438 (2013).

32. A. Oyagi, H. Hara, Essential roles of heparin-binding epidermal growth factor-like growth factor in the brain. CNS Neurosci. Ther. 18, 803–810 (2012).

33. R. B. Dorland, J. L. Middlebrook, S. H. Leppila, Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells. J. Biol. Chem. 254, 11337–11342 (1979).

34. T. Umata, Y. Moriyama, M. Futai, E. Mekada, The cytotoxic action of diphtheria toxin and its degradation in intact Vero cells are inhibited by bafilomycin A1, a specific inhibitor of vacuolar-type H+–ATPase. J. Biol. Chem. 265, 21940–21945 (1990).

35. A. Schulz, T. Ajayi, N. Specchio, E. de Los Reyes, P. Gissen, D. Ballon, J. P. Dyke, H. Cahan, P. Slasor, D. Jacoby, A. Kohlschutter; CLN2 Study Group, Study of intraventricular cerliponase alfa for CLN2 disease. N. Engl. J. Med. 378, 1989–1907 (2018).

36. L. Klinge, V. Straub, U. Neudorf, J. Schaper, T. Bösbach, K. Gorlinger, M. Wallot, S. Richards, T. Voit, Safety and efficacy of recombinant acid alpha-glucosidase (rhGAA) in patients with classical infantile Pompe disease: Results of a phase II clinical trial. Neuronmuscl. Disord. 15, 24–31 (2005).

37. D. P. Germain, J. Charrow, R. J. Desnick, N. Guffon, J. Kempf, R. H. Lachmann, R. Lemay, G. E. Linthorst, S. Packman, C. R. Scott, S. Waldek, D. G. Warnock, N. J. Weinreb, W. R. Wilcox, Ten-year outcome of enzyme replacement therapy with agalsidase beta in patients with Fabry disease. J. Med. Genet. 52, 353–358 (2015).

38. D. Vines, M. J. Warburton, Purification and characterisation of a tripeptidyl aminopeptidase I from rat spleen. Biochim. Biophys. Acta 1384, 233–242 (1998).

39. J. R. Crosby, C. Zhao, C. Jiang, D. Bai, M. Katz, S. Greenlee, H. Kawabe, M. Mc Caleb, D. Rotin, S. Guo, B. P. Monia, Inhaled ENaC antisense oligonucleotide ameliorates cystic fibrosis-like lung disease in mice. J. Cyst. Fibros. 16, 671–680 (2017).

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