Clearance of lysosomal glycogen accumulation by Transcription factor EB (TFEB) in muscle cells from lysosomal alpha-glucosidase deficient mice

Hoi Ming Li^{1,#}, Erin Feeney¹, Lishu Li¹, Hossein Zare¹, Rosa Puertollano², and Nina Raben¹

¹Laboratory of Muscle Stem Cells and Gene Regulation, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD, USA

²Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

Abstract

A recently proposed therapeutic approach for lysosomal storage disorders (LSDs) relies upon the ability of transcription factor EB (TFEB) to stimulate autophagy and induce lysosomal exocytosis leading to cellular clearance. This approach is particularly attractive in Glycogen Storage Disease type II (a severe metabolic myopathy and a paradigm for LSDs, also called Pompe disease) as the currently available therapy, replacement of the missing enzyme acid alpha-glucosidase, fails to reverse skeletal muscle pathology. Pompe disease is characterized by both lysosomal abnormality and dysfunctional autophagy. Here we show that TFEB is a viable therapeutic target in Pompe disease: overexpression of TFEB in a newly established conditionally immortalized skeletal muscle cell model reduced glycogen load and lysosomal size; and in the muscle fibers of GFP-LC3 Pompe disease mouse model significantly increased the motility of lysosomes in the fibers, and stimulated the fusion between lysosomes and autophagosomes under stress. Hence, modulation of TFEB activity holds promise for the development of a better therapy. In addition, the newly developed mouse and cell models have many potential applications such as large-scale drug screening for Pompe disease.

Keywords

Pompe disease; acid alpha-glucosidase; autophagy; lysosomal storage; myoblast immortalization; TFEB

Introduction

Glycogen Storage Disease type II, or Pompe disease (PD, OMIM: 232300), is a severe metabolic myopathy caused by the deficiency of acid alpha-glucosidase (GAA, acid maltase, E.C.3.2.1.20), an enzyme responsible for breaking down glycogen to glucose within the acidic environment of lysosomes. The functional deficiency or complete absence of the enzyme results in accumulation of glycogen within this cellular compartment [reviewed in

[#]Corresponding author: Hoi Ming Li, NIAMS, National Institutes of Health, Bethesda, MD, USA; Tel: +1 301 496 1949; hml.i.nih@gmail.com.

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[1,2]]. Although PD is a rare disorder, it shares common pathological features with many other lysosomal storage disorders (LSDs), which encompass more than sixty nosological entities [3].

Enzyme replacement therapy (ERT) is now available for PD patients; the therapy is aimed at replacing the missing enzyme with recombinant human GAA (rhGAA; alglucosidase alpha; Myozyme® and Lumizyme®, Genzyme Corporation, Framingham, MA). Clinical experience over the past decade provides evidence of both the efficacy and the limitations of this approach [4–7]. In infants, the therapy prevents death from cardiac failure but often leaves these patients with severe skeletal myopathy. Only modest, if any, improvement is observed in a number of patients with late-onset skeletal muscle myopathy [8].

Several factors contribute to the resistance of skeletal muscle to ERT. These include the sheer mass of muscle tissue, a relatively low density of the mannose-6-phosphate receptor that is responsible for the uptake of the enzyme, inefficient trafficking of the internalized enzyme to lysosomes, immune response to the recombinant enzyme in cross reacting material (CRM) -negative patients, and the presence of large areas of undigested autophagic debris alongside expanded lysosomes [9–13,7]. An entirely new concept of therapy, cellular clearance by induction of lysosomal exocytosis and biogenesis, has been recently put forward for LSDs and other disorders characterized by the accumulation of abnormal proteins [14]. It has been shown that the bHLH-leucine zipper transcription factor EB (TFEB) is a master regulator of lysosomal biogenesis and of autophagy [15–17]. Overexpression of TFEB in a variety of cell types leads to the generation of new lysosomes and increased numbers of autophagosomes and autophagolysosomes (also called autolysosomes), structures that are formed by the fusion of lysosomes and autophagosomes [16]. Furthermore, TFEB stimulates fusion of lysosomes not only with autophagosomes, but also with the plasma membrane, resulting in lysosomal exocytosis and cellular clearance in cell models of several LSDs [14].

Here, we have characterized the effect of TFEB overexpression on lysosomal and autophagic accumulation in both previously described and newly developed PD mouse and muscle cell models. Expression of TFEB in myotubes resulted in lysosomal exocytosis, and a dramatic reduction of intra-lysosomal glycogen accumulation. Furthermore, TFEB overexpression in PD muscle fibers significantly increased the movement of lysosomes in the fibers, and the formation of autophagolysosomes under stress. Thus, modulation of TFEB activity holds promise for the development of a better therapy for this devastating disorder.

Materials and Methods

Antibodies

rat anti-LAMP1 (1DB4) and mouse anti-MyoD (MoAb 5.8A) (both from BD Pharmingen, San Diego, CA); rabbit anti-LC3B and mouse anti-Flag (M2) (both from Sigma-Aldrich, St. Louis, MO); mouse anti-myogenin (F5D) (Dako, Glostrup, Denmark); mouse anti-myosin heavy chain (Clontech, Mountain View, CA); rabbit anti-p44/42 MAPK (Erk1/2), rabbit anti-phospho-p44/42 MAPK (Erk1/2; Thr202/Thr204), rabbit anti-phospho-4EBP1 (Thr37/46), and rabbit anti-histone H3 (D1H2) (Cell Signaling, Beverly, MA). Rabbit anti-alpha-tubulin (Abcam, Cambridge, MA) or mouse anti-vinculin antibody (Sigma-Aldrich) was used as a loading control for Western. Alexa Fluor-conjugated secondary antibodies (Life Technologies, Grand Island, NY) were used for immunostaining and Western analysis. Alexa Fluor 546 labeled recombinant human acid alpha-glucosidase (6.2 mg/ml) was provided by Genzyme Corp. (Framingham, MA).

Plasmids

plasmid containing the full-length 3xFlag-tagged human TFEB (referred to as Flag-TFEB) was described previously [15]; plasmid containing the full-length rat LAMP1 (mCherry-Lamp1) was obtained from Dr. Kristien Zaal (Light Imaging Section, Office of Science & Technology, NIAMS, NIH, Bethesda, MD, USA); plasmid containing GFP-TFEB was described previously [18].

Generation of mice and isolation of single live myofibers

The generation of GFP-LC3:GAA^{-/-}, immorto:GAA^{-/-}, and immorto:GFP-LC3:GAA^{-/-} mice is described in the Supporting Information. Single live myofibers from gastrocnemius and extensor digitorum longus (EDL) muscles from 3–4 month-old mice were isolated as described [19]. Isolation of single live fibers from flexor digitorum brevis (FDB) muscle was done with some modifications of the previously described protocol [13] (Supporting Information). Fibers were analyzed on a LSM 510 confocal microscope (Carl Zeiss, Göttingen, Germany; see Supporting Information) equipped with Plan-Neofluar 40X oil immersion objective.

In vivo electroporation of skeletal muscle and enzyme replacement therapy

Electric pulse-mediated gene transfer into FDB muscle was performed as described [20]. Three to four month-old GAA^{-/-} and GFP-LC3:GAA^{-/-} mice were used for the experiments (Supporting Information Table S1). The animals were sacrificed 4–6 days after the procedure. Live FDB fibers were isolated and analyzed by time lapse confocal microscopy. Alexa-Fluor 546 labeled recombinant human GAA (rhGAA) was administered into 3–4 month-old GFP-LC3:GAA^{-/-} mice at a dosage of 100 mg/kg twice with a 24 h interval. Mice were sacrificed 24 h after the last injections. Single fibers, isolated from EDL and gastrocnemius muscle, were analyzed by confocal microscopy.

Fibroblast and myoblast culture systems

Primary skin fibroblasts were derived from two unrelated patients with severe infantile PD. Clinical diagnosis was confirmed by genetic analysis. Fibroblasts were maintained in DMEM (high glucose) supplemented with 10% (w/v) FBS, 1X P/S/L-Glutamine. Adult mouse fibroblasts were isolated from the tail tip according to a standard procedure.

Immortalized PD cell lines were derived from immorto:GAA^{-/-} and immorto:GFP-LC3:GAA^{-/-} mice. Single fibers from EDL muscle of 3–4 month-old mice were plated in each well (~10 fibers per well) of a Matrigel-coated 6-well plate in plating medium (10% horse serum in DMEM, 0.5% chick embryo extract, and 1X P/S/L-Glutamine) at 37 °C, 5% CO₂. After 3 days, when the satellite cells began to migrate away from fibers, most of the medium (3/4) was replaced with proliferation medium (20% fetal bovine serum, 10% horse serum, 1% chick embryo extract, 1X P/S/L-Glutamine in high glucose DMEM). Myogenic colonies were isolated with cloning cylinders (Corning, Corning, NY) on day 5 or 6. Several individual myogenic clones were isolated and analyzed for their proliferation capacity and the ability to differentiate into myotubes. To induce the expression of H-2K^b-tsA58 SV40 large T-antigen for immortalization of the satellite cells and myoblasts, recombinant IFN- (Life Technologies) was added to the proliferation medium (100 units/ml). The cells were then incubated at 33 °C in an atmosphere of 5% CO₂ [21]. When myoblasts became nearly confluent, the medium was changed to differentiation medium (DMEM containing 2% horse serum, 0.5% chick embryo extract, 1X P/S/L-Glutamine) and the cells were moved to 37 °C in an atmosphere of 5% CO₂. Myotubes began to form within two days. Glucose

concentration was either 1 g/L (low) or 4.5 g/L (high). Myotubes survived in culture for about 3 weeks. A wild type immortalized muscle cell line served as the control.

Infection of myotubes with adenovirus expressing TFEB and immunofluorescence microscopy

Adenoviruses expressing either wild-type or constitutively active mutant (S211A) TFEB were described previously [18]. Myotubes were infected with either adenovirus (Ad-null) or adenovirus expressing wild-type TFEB (Ad-TFEB) or S211A TFEB (Ad-TFEBmt) for 24, 48, or 72 hours. Myotubes were fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min at room temperature, washed twice in PBS, and permeabilized in 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO). Immunostaining with LAMP1, LC3, or Flag antibodies was done using M.O.M. kit (Vector Laboratories, Burlingame, CA) as previously described [22]. The cell nuclei were stained with 2 µg/mL Hoechst 33342 (Life Technologies) in PBS for 10 min. After staining, the cells were imaged on a Carl Zeiss LSM 510 confocal microscope with an oil immersion objective.

Fluorescent glycogen detection, surface LAMP1 analysis, and in situ detection of apoptotic cells

Lysosomal glycogen in live cells was detected by the incorporation of 2-NBDG, a D-glucose fluorescent derivative (2-deoxyglucose), into glycogen as described [23]. Surface LAMP assay was done as described [14]. Apoptotic Cells were detected with TUNEL using the ApopTag® Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore, Billerica, MA).

Quantitative analyses are presented in the Supporting Information. Data in text are given as mean ± S.E.. Kolmogorov-Smirnov test was used for comparison of cumulative distributions of lysosomal size in myotubes; Wilcoxon rank sum test was used for comparison of median values. Student's *t*-test was used for all other comparisons. Differences were considered significant at $p < 0.05$.

Animal care and experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the European Union Directive 86/609 regarding the protection of animals used for experimental purposes.

Results

Establishment and characterization of new *in vitro* and *in vivo* PD models

To test new therapeutic approaches for PD, we established conditionally immortalized skeletal myogenic cells (Supporting Information, Fig. S1). PD myotubes but not myoblasts or fibroblasts (Supporting Information, Fig. S2) replicated lysosomal pathology, namely the enlargement of lysosomes and abnormal glycogen storage (Fig. 1A and D). Disappointingly, the secondary abnormality in PD muscle fibers, autophagic accumulation [reviewed in [12]], was not reproduced in PD myotubes as demonstrated by immunostaining and Western analysis with LC3 [a highly specific autophagosomal marker [24]] antibodies (shown for Western in Supporting Information Fig. S1D).

In contrast, autophagic pathology was clearly visible in muscle fibers derived from a newly developed PD mouse model, in which autophagosomes were labeled with GFP-LC3 (GFPLC3:GAA^{-/-}). In this new strain, large areas of autophagic accumulation can be seen in live myofibers without staining (Fig S3). This buildup posed an obstacle for ERT: when labeled rhGAA was administered intravenously in these mice, the drug was detected almost

exclusively within autophagosomes clustered in the buildup areas (Fig. S3). In an attempt to uncover any subtle abnormalities in autophagy in our cell culture system, we established myoblast cells from GFP-LC3:GAA^{-/-} mice as well. However, no autophagic accumulation was observed in myotubes in these lines, although basal autophagy was functional as evidenced by the response to starvation and bafilomycin (Supporting Information Fig. S4). Thus, the cell culture system can only mimic the lysosomal defects of PD, but not autophagic abnormalities.

TFEB overexpression reduced lysosomal size and glycogen burden in PD myotubes

To see if TFEB can promote lysosomal exocytosis and rescue lysosomal glycogen storage in multinucleated muscle cells, PD myotubes were infected with adenovirus expressing Flag-TFEB (Ad-TFEB), followed by fixation and immunostaining with anti-LAMP1 (lysosomal marker) and anti-Flag antibodies.

Robust expression and nuclear staining of TFEB in myotubes were achieved after 48–72 hours and resulted in a dramatic reduction of lysosomal size ($p=6.32 \times 10^{-8}$; Fig. 1A and B; Supporting Information Fig. S5A). PD myotubes infected with the adenovirus control vector (Ad-null) showed large LAMP1-positive lysosomes similar to those seen in non-infected cells (Fig. 1A). Earlier, at 24 hours post-infection, TFEB-expressing cells (~10–20% of myotubes) showed a striking relocation of enlarged lysosomes toward the plasma membrane; images taken at this time point provide a snapshot of the process of lysosomal secretion (Fig. 1C, top). Lysosomal exocytosis was confirmed by the surface LAMP assay showing the presence of lysosomal membrane marker on the plasma membrane in TFEB-expressing myotubes (Fig. 1C, lower) but not in non-infected cells (Fig. 1C, middle). TFEB also stimulated autophagy in PD myotubes, as evidenced by an increase in autophagosomes and LC3 level (Supporting Information Fig. S5B and C).

In addition, we tested the effect of constitutively active mutant TFEB (S211A; TFEBmt) [18,25,26] in PD myotubes. Massive accumulation of TFEB in the nuclei resulted in a striking clearance of large lysosomes without appreciable changes in the total amount of LAMP protein, consistent with the role of TFEB in lysosomal biogenesis [15,16] (Fig. 2A and B).

As expected, the removal of enlarged lysosomes from PD myotubes was associated with a significant decrease in the amount of accumulated storage material, as shown by the incorporation of the fluorescent glucose derivative 2-NBDG into glycogen (Fig. 1D). Thus, muscle cells in Pompe disease can be cleared of lysosomal glycogen accumulation by TFEB induction. However, one caveat is that many TFEB-overexpressing muscle cells changed their morphology: normally elongated myotubes became spindle-like, contain centrally located nuclei, and some underwent apoptosis (Fig. 1A, 2A and S6).

It has been recently shown that inhibition of the master growth regulator mTOR complex 1 (mTORC1) activates TFEB by promoting its nuclear translocation [18,25,26]. Therefore, we attempted to activate endogenous TFEB by pharmacologically targeting mTORC1. Addition of Torin 1 and Torin 2 inhibited mTORC1 [27] and stimulated autophagy, but failed to reduce lysosomal size (Supporting Information, Fig. S7A and B; shown for Torin 2). These data suggest that the amount of endogenous TFEB may not be sufficient to support lysosomal clearance; alternatively, another kinase, MAPK (ERK1/2) kinase, may be involved in the process of TFEB activation [17]. A striking increase in the levels of p-ERK1/2 in TFEB-treated PD myotubes points to such a possibility in muscle cells (Supporting Information Fig. S7C).

TFEB overexpression in PD muscle induced cellular clearance by promoting lysosomal/autophagosomal exocytosis

To evaluate the effect of TFEB on muscle tissue *in vivo*, two knockout mouse strains were used: the previously described GAA^{-/-} [13] and a newly developed GFP-LC3:GAA^{-/-} strain. The use of these two models allowed us to explore the consequences of TFEB overexpression not only on lysosomal but also on autophagic pathology [12]. FDB muscles from each line were transfected by electroporation with different combinations of plasmids containing TFEB and LAMP (Table S1). Four to six days after transfection, live single muscle fibers were analyzed by time-lapse microscopy. The efficiency of the transfection in all conditions ranged from 10 to 30%.

Both Flag-TFEB/mCherry-LAMP and GFP-TFEB/mCherry-LAMP-transfected PD fibers (Table S1, conditions 2 and 3) showed a markedly increased motility of lysosomes (Fig. 3, S8; Movie 1 and 4) compared to untreated PD controls (Table S1, condition 1; Movie 2). The mean maximum velocity of lysosomes in TFEB-treated fibers was $0.247 \pm 0.01 \mu\text{m}/\text{min}$ [pooled data from conditions 2 (n=24) and 3 (n=19)], whereas this value was $0.130 \pm 0.006 \mu\text{m}/\text{min}$ (n=26) in untreated controls ($p = 2.07 \times 10^{-17}$). A vast majority of TFEB transfected fibers maintained normal shape and appearance, suggesting that overexpression of TFEB did not cause any appreciable muscle damage. However, stress of fibers exposed to hours of time-lapse confocal microscopy resulted in a massive translocation of TFEB into the nuclei, and occasionally, the detachment of plasma membrane occurred afterwards (Fig. 3A, arrow; Supporting Information Fig. S9A; Movie 3). This phenomenon was not seen in control experiments, in which the muscle of GFP-LC3:GAA^{-/-} mice was transfected with mCherry-LAMP only (Table S1, condition 1).

The choice of the GFP-LC3:GAA^{-/-} strain for control experiments enabled us not only to evaluate the motility of lysosomes, but also to examine the possibility of impaired lysosomal/autophagosomal fusion as a mechanism of PD. Unexpectedly, the expression of mCherry-LAMP in most transfected fibers was seen almost exclusively in fibers or in regions of fibers free from autophagic buildup (~80–90% of all transfected fibers exhibited these two expression patterns in roughly equal proportions; Supporting Information Fig. S9B), suggesting a compromised *de novo* lysosomal biogenesis or transportation in areas of autophagic accumulation. Hence, to analyze the effect of TFEB on lysosomal-autophagosomal fusion, we took advantage of stress-induced surges in the number of LC3-positive autophagosomes during long hours of time-lapse microscopy. During these surges, TFEB-treated fibers (Table S1, condition 2) exhibited a striking increase in lysosomal-autophagosomal fusion compared to buildup free control fibers (transfected with mCherry-LAMP only; condition 1; Movie 2), as indicated by co-localization of LAMP (red) and LC3 (green) markers (Fig. 3C, D; Movie 4). Furthermore, the transfected fibers showed alignment of doubly labeled autophagolysosomes (rather than vesicles labeled by LAMP only) along the plasma membrane (Fig. 3E), suggesting that TFEB may induce lysosomal/autophagosomal exocytosis. These data raised an intriguing possibility that autophagy may facilitate TFEB-induced lysosomal clearance.

Discussion

Defects in autophagy significantly contribute to the pathophysiology of several lysosomal storage diseases [28,29,3,30,31,12] including PD, which has particularly striking autophagic buildup. Autophagic accumulation hinders the delivery of the therapeutic enzyme to lysosomes. Consistent with our previous observations *in vitro* [32], we here provide direct evidence that in ERT the bulk of the intravenously administered rhGAA drug ends up not in lysosomes but in LC3-positive autophagosomes within the area of buildup. Hence, it is necessary to develop new therapies for PD.

Inspired by the recent discoveries of cellular clearance of abnormal protein accumulation in LSDs and other disorders through overexpression of TFEB [14–17], here in this report, we have shown that TFEB efficiently triggers lysosomal exocytosis and promotes cellular clearance of glycogen-filled lysosomes in PD myotubes, and facilitated the formation of autophagolysosome in isolated live PD muscle fibers under stressful condition. All these provided strong evidence of TFEB's potential as a therapeutic target in PD.

The simplicity of detecting hugely expanded lysosomes and glycogen in PD myotubes made the cell model particularly attractive: the effects of TFEB were easily monitored and evident within days. In TFEB-treated myotubes, the lysosomal marker LAMP, a protein typically present inside the cells, was seen on the outer plasma membrane, indicating that lysosomal clearance proceeds through exocytosis in muscle cells. Furthermore, the cell system allowed us to expose a potential problem with high TFEB activity; a constitutively active form of TFEB, in which S211 is mutated to alanine [18], invariably resulted in morphological changes of myotubes, perhaps because of massive expulsion of the lysosomes.

TFEB-treated fibers exhibited a dramatic increase in the motility of lysosomes and the fusion between LC3- and LAMP-positive vesicles, an appearance of multiple LC3/LAMP-positive autophagolysosomes, and docking of these double-positive structures to the plasma membrane. These data suggest that TFEB may prevent autophagic buildup by stimulating the formation and secretion of autophagolysosomes, a product of lysosomal-autophagosomal fusion. Hence, it is worthful to have further long term experiments such as *in vivo* TFEB intramuscular delivery with adeno-associated virus in PD mice to see whether the autophagic and lysosomal pathologies in PD muscle can be corrected by TFEB constitutive expression and inducible activation.

Finally, the potential applications of our newly developed models, PD myotubes and GFPLC3:GAA $-/-$ mice, extend beyond testing the effects of TFEB. For example, an *in vitro* PD model is much needed for large-scale drug testing. Recently, several attempts have been made to develop such a model: these include the generation of cardiomyocyte-like cells from human induced pluripotent stem (iPS) cells [33], muscle cells from reprogrammed murine iPS cells [34], and murine muscle cells transduced by viral constructs containing immortalizing genes [35,36]. Our new PD muscle cell lines have several advantages: they are highly myogenic, derived from individual clones, and, most importantly, they maintain the capacity to differentiate when the immortalizing gene, shown to perturb myogenic program, is inactivated [35,37]. Therefore, these new mouse and cell models would certainly much benefit the scientific community of PD research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Kroos M, Hoogeveen-Westerveld M, van der Ploeg A, Reuser AJJ. The genotype-phenotype correlation in Pompe disease. *Am J Med Genet C Semin Med Genet.* 2012; 160:59–68. [PubMed: 22253258]
2. van der Ploeg AT, Reuser AJJ. Pompe's disease. *Lancet.* 2008; 372:1342–1353. [PubMed: 18929906]
3. Cox TM, Cachón-González MB. The cellular pathology of lysosomal diseases. *J Pathol.* 2012; 226:241–254. [PubMed: 21990005]
4. Van den Hout JMP, Kamphoven JHJ, Winkel LPF, Arts WFM, De Klerk JBC, Loonen MCB, et al. Long-term intravenous treatment of Pompe disease with recombinant human alpha-glucosidase from milk. *Pediatrics.* 2004; 113:e448–457. [PubMed: 15121988]
5. Kishnani PS, Corzo D, Nicolino M, Byrne B, Mandel H, Hwu WL, et al. Recombinant human acid [alpha]-glucosidase: major clinical benefits in infantile-onset Pompe disease. *Neurology.* 2007; 68:99–109. [PubMed: 17151339]
6. Strothotte S, Strigl-Pill N, Grunert B, Kornblum C, Eger K, Wessig C, et al. Enzyme replacement therapy with alglucosidase alfa in 44 patients with late-onset glycogen storage disease type 2: 12-month results of an observational clinical trial. *J Neurol.* 2010; 257:91–97. [PubMed: 19649685]
7. van der Ploeg AT, Clemens PR, Corzo D, Escolar DM, Florence J, Groeneveld GJ, et al. A randomized study of alglucosidase alfa in late-onset Pompe's disease. *N Engl J Med.* 2010; 362:1396–1406. [PubMed: 20393176]
8. Schoser B, Hill V, Raben N. Therapeutic approaches in glycogen storage disease type II/Pompe Disease. *Neurotherapeutics.* 2008; 5:569–578. [PubMed: 19019308]
9. Banugaria SG, Prater SN, Ng YK, Kobori JA, Finkel RS, Ladda RL, et al. The impact of antibodies on clinical outcomes in diseases treated with therapeutic protein: lessons learned from infantile Pompe disease. *Genet Med.* 2011; 13:729–736. [PubMed: 21637107]
10. Funk B, Kessler U, Eisenmenger W, Hansmann A, Kolb HJ, Kiess W. Expression of the insulin-like growth factor-II/mannose-6-phosphate receptor in multiple human tissues during fetal life and early infancy. *J Clin Endocrinol Metab.* 1992; 75:424–431. [PubMed: 1379254]
11. Koeberl DD, Li S, Dai J, Thurberg BL, Bali D, Kishnani PS. 2 Agonists enhance the efficacy of simultaneous enzyme replacement therapy in murine Pompe disease. *Mol Genet Metab.* 2012; 105:221–227. [PubMed: 22154081]
12. Raben N, Wong A, Ralston E, Myerowitz R. Autophagy and mitochondria in Pompe disease: nothing is so new as what has long been forgotten. *Am J Med Genet C Semin Med Genet.* 2012; 160:13–21. [PubMed: 22253254]
13. Raben N, Schreiner C, Baum R, Takikita S, Xu S, Xie T, et al. Suppression of autophagy permits successful enzyme replacement therapy in a lysosomal storage disorder--murine Pompe disease. *Autophagy.* 2010; 6:1078–1089. [PubMed: 20861693]
14. Medina DL, Fraldi A, Bouche V, Annunziata F, Mansueto G, Spampanato C, et al. Transcriptional activation of lysosomal exocytosis promotes cellular clearance. *Dev Cell.* 2011; 21:421–430. [PubMed: 21889421]
15. Sardiello M, Palmieri M, di Ronza A, Medina DL, Valenza M, Gennarino VA, et al. A gene network regulating lysosomal biogenesis and function. *Science.* 2009; 325:473–477. [PubMed: 19556463]
16. Settembre C, Ballabio A. TFEB regulates autophagy: an integrated coordination of cellular degradation and recycling processes. *Autophagy.* 2011; 7:1379–1381. [PubMed: 21785263]
17. Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, et al. TFEB links autophagy to lysosomal biogenesis. *Science.* 2011; 332:1429–1433. [PubMed: 21617040]
18. Martina JA, Chen Y, Gucek M, Puertollano R. MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. *Autophagy.* 2012; 8:903–914. [PubMed: 22576015]
19. Rosenblatt JD, Lunt AI, Parry DJ, Partridge TA. Culturing satellite cells from living single muscle fiber explants. *In Vitro Cell Dev Biol Anim.* 1995; 31:773–779. [PubMed: 8564066]

20. DiFranco M, Quinonez M, Capote J, Vergara J. DNA transfection of mammalian skeletal muscles using in vivo electroporation. *J Vis Exp*. 2009
21. Jat PS, Noble MD, Ataliotis P, Tanaka Y, Yannoutsos N, Larsen L, et al. Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. *Proc Natl Acad Sci USA*. 1991; 88:5096–5100. [PubMed: 1711218]
22. Raben N, Shea L, Hill V, Plotz P. Monitoring autophagy in lysosomal storage disorders. *Meth Enzymol*. 2009; 453:417–449. [PubMed: 19216919]
23. Louzao MC, Espiña B, Vieytes MR, Vega FV, Rubiolo JA, Baba O, et al. “Fluorescent glycogen” formation with sensibility for in vivo and in vitro detection. *Glycoconj J*. 2008; 25:503–510. [PubMed: 17973187]
24. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J*. 2000; 19:5720–5728. [PubMed: 11060023]
25. Rocznik-Ferguson A, Petit CS, Froehlich F, Qian S, Ky J, Angarola B, et al. The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. *Sci Signal*. 2012; 5:ra42. [PubMed: 22692423]
26. Settembre C, Zoncu R, Medina DL, Vetrini F, Erdin S, Erdin S, et al. A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J*. 2012; 31:1095–1108. [PubMed: 22343943]
27. Liu Q, Chang JW, Wang J, Kang SA, Thoreen CC, Markhard A, et al. Discovery of 1-(4-(4-propionylpiperazin-1-yl)-3-(trifluoromethyl)phenyl)-9-(quinolin-3-yl)benzo[h][1,6]naphthyridin-2(1H)-one as a highly potent, selective mammalian target of rapamycin (mTOR) inhibitor for the treatment of cancer. *J Med Chem*. 2010; 53:7146–7155. [PubMed: 20860370]
28. Angelini C, Semplicini C. Enzyme replacement therapy for Pompe disease. *Curr Neurol Neurosci Rep*. 2012; 12:70–75. [PubMed: 22002767]
29. Cao Y, Espinola JA, Fossale E, Massey AC, Cuervo AM, MacDonald ME, et al. Autophagy is disrupted in a knock-in mouse model of juvenile neuronal ceroid lipofuscinosis. *J Biol Chem*. 2006; 281:20483–20493. [PubMed: 16714284]
30. Liao G, Yao Y, Liu J, Yu Z, Cheung S, Xie A, et al. Cholesterol accumulation is associated with lysosomal dysfunction and autophagic stress in *Npc1* $-/-$ mouse brain. *Am J Pathol*. 2007; 171:962–975. [PubMed: 17631520]
31. Lieberman AP, Puertollano R, Raben N, Slaugenhaupt S, Walkley SU, Ballabio A. Autophagy in lysosomal storage disorders. *Autophagy*. 2012; 8:719–730. [PubMed: 22647656]
32. Fukuda T, Ahearn M, Roberts A, Mattaliano RJ, Zaal K, Ralston E, et al. Autophagy and mistargeting of therapeutic enzyme in skeletal muscle in Pompe disease. *Mol Ther*. 2006; 14:831–839. [PubMed: 17008131]
33. Huang HP, Chen PH, Hwu WL, Chuang CY, Chien YH, Stone L, et al. Human Pompe disease-induced pluripotent stem cells for pathogenesis modeling, drug testing and disease marker identification. *Hum Mol Genet*. 2011; 20:4851–4864. [PubMed: 21926084]
34. Kawagoe S, Higuchi T, Meng XL, Shimada Y, Shimizu H, Hirayama R, et al. Generation of induced pluripotent stem (iPS) cells derived from a murine model of Pompe disease and differentiation of Pompe-iPS cells into skeletal muscle cells. *Mol Genet Metab*. 2011; 104:123–128. [PubMed: 21703893]
35. Douillard-Guilloux G, Mouly V, Caillaud C, Richard E. immortalization of murine muscle cells from lysosomal alpha-glucosidase deficient mice: a new tool to study pathophysiology and assess therapeutic strategies for Pompe disease. *Biochem Biophys Res Commun*. 2009; 388:333–338. [PubMed: 19665008]
36. Takikita S, Myerowitz R, Zaal K, Raben N, Plotz PH. Murine muscle cell models for Pompe disease and their use in studying therapeutic approaches. *Mol Genet Metab*. 2009; 96:208–217. [PubMed: 19167256]
37. Mouly V, Edom F, Decary S, Vicart P, Barbert JP, Butler-Browne GS. SV40 large T antigen interferes with adult myosin heavy chain expression, but not with differentiation of human satellite cells. *Exp Cell Res*. 1996; 225:268–276. [PubMed: 8660914]

Highlights

- GFP-LC3 Pompe disease (PD) mice and immortalized murine muscle cells were generated
- Overexpression of TFEB in the PD myotubes reduced lysosomal glycogen buildup
- Overexpression of TFEB in the PD muscle fibers increased the motility of lysosomes
- Stress-induced activation of TFEB promoted autolysosome formation in PD fibers
- Hence, TFEB is a potentially viable therapeutic target in PD

Figure 1. TFEB stimulated clearance of enlarged lysosomes and reduced glycogen burden in PD myotubes

A. Confocal microscopy images of wild-type (WT) myotubes (left) and PD myotubes (cl. 3LE8) infected for 72 hours with either adenovirus (Ad-null) or adenovirus containing TFEB (Ad-TFEB) showed a dramatic reduction in the number of large LAMP1-positive lysosomes (red). Nuclei were stained with Hoechst (blue). TFEB was detected with anti-Flag antibody (green).

B. Distribution of lysosomal size in Ad-null and Ad-TFEB PD myotubes. Lysosomal size is expressed as number of pixels representing lysosomal area (LAMP-positive structures). The median lysosomal size of Ad-TFEB infected myotubes ($m=367.13$ pixels, $n=703$, range 2082659) was significantly lower than that of Ad-null infected myotubes ($m=491.16$ pixels, $n=1395$, range 200–2857; $p=6.5 \times 10^{-12}$).

C. Confocal microscopy image of PD myotubes infected for 24 hours with Ad-TFEB show relocation of lysosomes to the plasma membrane (top). Images showing Lamp1 staining (red) on plasma membrane in a PD myotube infected with Ad-TFEB (bottom; arrows) but not in a non-infected cell (middle). Non-permeabilized cells were incubated with anti-LAMP1 antibody at 4°C for 40 min, followed by fixation and staining with secondary antibody.

D. Confocal microscopy images of live non-infected PD myotubes (left) or PD myotubes infected for 72 hours with Ad-TFEB (right) showed a dramatic reduction in the amount of accumulated glycogen, stained by 2-NBDG. Bar: 10 μ m.

Figure 2. TFEBmt reduced lysosomal size in PD myotubes

A. Immunostaining of the control and myotubes infected with a mutant form of TFEB (TFEBmt), with LAMP (red), Flag (green) and Hoechst (blue). Ad-null or Ad-TFEBmt were added to the myotubes respectively for 72 hours on day 11 in differentiation medium. Ad-TFEBmt infected cells showed massive accumulation of TFEB in the nuclei and significant reduction in lysosomal size, similar to wild-type TFEB. Bar: 10 μ m.

B. Western blot of cell lysates confirmed the translocation of TFEB in the nuclear fraction; TFEBmt treatment did not affect the total amount of LAMP protein, consistent with the role of TFEB in lysosomal biogenesis. The different intensities of the two bands corresponding to LAMP protein in untreated and TFEBmt-treated samples may reflect the differences in the glycosylation pattern.

Figure 3. TFEB increased lysosomal motility in PD fibers and stimulated lysosomal-autophagosomal fusion under stress

A. FDB muscle of a GAA^{-/-} mouse was transfected with both GFP-TFEB (green) and mCherry-LAMP (red) (GAA^{-/-} + GFP-TFEB). Confocal images were taken before (left) and after four hours (right) of time-lapse microscopy. After 4 hours of time-lapse microscopy, a surge of TFEB was activated as evidenced by its sudden nuclear translocation (green nuclei).

B. The mean maximum velocity of lysosomes in untreated and TFEB-transfected PD fibers (note: all data for Flag- and GFP-TFEB-treated fibers are pooled). Lysosomal velocities were calculated from time-lapse images using ImageJ software with the manual tracking plug-in. For each condition the trajectories of multiple lysosomes were followed (n=26 untreated; n=43 TFEB-treated) and the three highest velocity measurements per lysosome were recorded. In TFEB-transfected fibers, the maximum velocity of lysosomes was significantly increased ($p=2.07 \times 10^{-17}$). * indicates statistically significant differences ($p < 0.001$). Error bars represent 95% confidence intervals.

C–E. Images of live muscle fibers from GFP-LC3:GAA^{-/-} mice after long hours of time-lapse confocal microscopy:

(C) Muscle was transfected with mCherry-LAMP only. The image (a single frame from the time-lapse series presented in Movie 2) shows lysosomes (red), LC3-positive autophagosomes (green) and a number of autolysosomes (yellow).

(D) Muscle was transfected with Flag-TFEB and mCherry-LAMP. Massive formation of autolysosomes is indicated by yellow structures.

(E) A snapshot of the process of exocytosis. The structures at the plasma membrane were labeled with both LC3 and LAMP, indicating that they represented amphisomes (a product of fusion between autophagic vesicles and late endosomes) or autolysosomes (a product of fusion between autophagic vesicles and lysosomes). Bar: 10μm for all panels.