Pompe Disease Results in a Golgi-based Glycosylation Deficit in Human Induced Pluripotent Stem Cell-derived Cardiomyocytes

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*Running title: Golgi glycosylation defect in Pompe IPSC-cardiomyocytes

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Background: How the absence of lysosomal enzyme acid α-glucosidase causes hypertrophic cardiomyopathy in Pompe disease is unknown.

Results: Pompe patient induced pluripotent stem cell-derived cardiomyocytes have normal autophagic and contractile function, but exhibit a deficit of golgi-based protein glycosylation.

Conclusions: Loss of lysosomal glycogen hydrolyzing ability results in protein glycosylation deficits.

Significance: Malfunctioning proteins due to mis-glycosylation may contribute to the pathophysiology of Pompe cardiomyopathy.

ABSTRACT

Infantile onset Pompe disease is an autosomal recessive disorder caused by the complete loss of lysosomal glycogen hydrolyzing enzyme acid α-glucosidase (GAA) activity, which results in lysosomal glycogen accumulation and prominent cardiac and skeletal muscle...
pathology. The mechanism by which loss of GAA activity causes cardiomyopathy is poorly understood. We reprogrammed fibroblasts from patients with infantile-onset Pompe disease to generate induced pluripotent stem (iPS) cells that were differentiated to cardiomyocytes (iPSC-CMs). Pompe iPSC-CMs had undetectable GAA activity and pathognomonic glycogen-filled lysosomes; nonetheless, Pompe and Control iPSC-CMs exhibited comparable contractile properties in engineered cardiac tissue. Impaired autophagy has been implicated in Pompe skeletal muscle; however, Control and Pompe iPSC-CMs had comparable clearance rates of LC3II-detected autophagosomes. Unexpectedly, the lysosomal associated membrane proteins, LAMP1 and LAMP2, from Pompe iPSC-CMs demonstrated higher electrophoretic mobilities compared to control iPSC-CMs. Brefeldin A induced disruption of the golgi in Control iPSC-CMs reproduced the higher mobility forms of the LAMPs, suggesting that Pompe iPSC-CMs produce LAMPs lacking appropriate glycosylation. Isoelectric focusing studies revealed that LAMP2 has a more alkaline pI in Pompe compared to control iPSC-CMs due largely to hypo-sialylation. MALDI-TOF-MS analysis of N-linked glycans demonstrated reduced diversity of multi-antennary structures and the major presence of a tri-mannose complex glycan precursor for Pompe iPSC-CMs. These data suggest that Pompe cardiomyopathy has a glycan processing abnormality and thus shares features with hypertrophic cardiomyopathies observed in the congenital disorders of glycosylation.

Infantile-onset Pompe disease is an autosomal recessive glycogen storage disorder caused by the complete loss of acid α-glucosidase (GAA) activity, the sole enzyme responsible for hydrolyzing glycogen into free glucose within the lysosome. Without GAA activity, glycogen accumulates within the lysosomes and is associated with prominent pathological manifestations in skeletal and cardiac muscle. The disease presents during the first days to months of life and without enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA), patients die within 1 year due to respiratory insufficiency or cardiac complications including arrhythmias and heart failure (1,2). ERT represents a major breakthrough prolonging survival, but the treatment is not curative and has significant limitations. For example, patients can develop immune reactivity to the intravenously administered enzyme (3). Skeletal muscle often becomes refractory to treatment due to endosomal and autophagic sequestration of rhGAA, preventing rhGAA from reaching the lysosomes which results in the re-accumulation of glycogen (4). Although the cardiomyopathy can be suppressed indefinitely if treatment is initiated early, long-term treatment is complicated by arrhythmias (5,6). Thus, there remains significant need to further advance the understanding of Pompe pathophysiology in order to improve treatment strategies.

The mechanisms by which lack of GAA activity and lysosomal glycogen accumulation cause muscle pathology have been investigated primarily in skeletal muscle. Electron micrographs of patient skeletal muscle demonstrate a progressive replacement of contractile structures with glycogen-filled lysosomes and free glycogen (7). Moreover, both intact and skinned muscle fibers from the Pompe mouse model produce less force, compared to healthy controls, per unit muscle mass (8). While these data provide some explanation for the global skeletal muscle weakness, mechanisms leading to the early cardiac phenotype of hypertrophic cardiomyopathy are largely unknown.

Autophagic dysfunction has been implicated as an upstream pathology leading to the skeletal muscle wasting (9,10). Pompe skeletal muscle has accumulations of autophagosomes and ubiquitinated protein aggregates, indicating that the lysosomes are not digesting intracellular materials at a sufficient rate (11). The observation that glycogen-filled lysosomes have neutral pH supports the argument for lysosomal incompetence (12). Lysosomal rupture and insufficient clearance of old/damaged mitochondria (mitophagy) are hypothesized to be direct mechanisms of contractile protein loss via release of acid and hydrolytic enzymes into the cytosol and increased mitochondrial reactive oxygen species production (13).

In cardiac muscle the pathology differs with development of hypertrophy rather than
atrophy. Whether autophagy is central to cardiac pathophysiology is unknown (14). Pompe cardiomyopathy is classified as a hypertrophic cardiomyopathy (HCM) because of the marked thickening of the ventricular walls and associated hyperdynamic systolic function with outflow tract obstruction. These features are shared with familial HCM due to mutations in the myosin heavy chain genes or other myofilament genes in which abnormalities in contractile properties are thought to induce hypertrophy. Whether Pompe cardiomyocytes exhibit abnormalities in contractile function has not yet been directly investigated (15,16).

In this study, we generated human induced pluripotent stem (iPS) cells from two different patients with infantile Pompe disease and differentiated them to iPS cell-derived cardiomyocytes (iPSC-CMs) as a model to study the cardiac manifestations of Pompe disease. The Pompe iPSC-CMs exhibited undetectable GAA activity and pathognomonic glycogen-filled lysosomes. Engineered cardiac tissue (ECT) generated from Pompe and Control iPSC-CMs demonstrated no consistent differences in contractile strength or kinetics. Moreover, Pompe iPSC-CMs were capable of lysosomal digestion of autophagosomes to the same extent as controls. However, we observed differences in electrophoretic mobilities and isoelectric points of lysosomal associated membrane proteins (LAMPs) from Pompe iPSC-CMs in comparison to control iPSC-CMs. We provide evidence of Pompe iPSC-CM specific deficits in LAMP1 and LAMP2 glycosylation as well as global deficits in N-linked glycan synthesis that occur in the Golgi. We propose that infantile Pompe disease may share disease mechanisms with other congenital disorders of glycosylation that have similar Golgi-based glycosylation deficiencies and also have clinical presentations that include hypertrophic cardiomyopathy and muscle weakness early in life (17,18).

**EXPERIMENTAL PROCEDURES**

*Generation of iPS cells from skin fibroblasts –* Patient fibroblasts (I.D. GM20089, GM20123, and GM04912) were purchased from the Coriell Institute for Medical Research and reprogrammed into iPS cells using the 6-factor (OCT4, NANOG, SOX2, KLF4, LIN28, and cMYC) lentiviral transfection protocol (19). The iPS cells were cultured with the TeSR/Matrigel system (20), verified as pluripotent with teratoma assays in SCID/beige mice, and Giemsa-band karyotyped before aliquots were frozen for later experimentation.

*Acid α-glucosidase genotyping, activity assay, and immunodetection –* Pompe line GM04912 gDNA was PCR amplified to evaluated for disease mutations with primers in table 1 and Sanger sequenced. The Pompe line GM20089’s exon 18 deletion was amplified with primers in table 1 and the products were visualized on agarose gel. Acid glucosidase activity was measured by the hydrolysis of 4-methylumbelliferyl-α-D-glucoside (4-MUG, Sigma) at pH=4 to release the fluorophore (4-MU) as previously described (21). GAA protein from iPS cells was immunoblotted with rabbit polyclonal anti-GAA (gift from Duke Glycogen Storage Disease Laboratories) by using the 1D western blotting procedure below.

**Dissociation of iPSC cells into cardiomyocytes –** The iPSC cell lines were differentiated into cardiomyocytes using a small molecule directed differentiation protocol in a 12-well plate format (22). Once differentiated, cardiomyocytes were maintained in RPMI medium (Gibco) supplemented with B27 (Gibco) (RPMI/B27). At 30 days following initiation of differentiation, cells were dissociated with 10x TryPLE (Gibco) as clumps and re-plated onto gelatin coated T75 flasks in DMEM/20% FBS for two days and then maintained for another 8 days in RPMI/B27. Cells were then dissociated to single cells with 10x TryPLE and either: 1) used for engineered cardiac tissue (ECT) formation as described in the next section, 2) replated onto gelatin coated 12 well plates (first 2 days in DMEM/20% FBS followed by RPMI/B27) at 1 million cells/well for protein studies, or 3) plated at a variety of densities on glass coverslips for immunocytochemistry. For electron microscopy, contracting regions were microdissected from original differentiation plates and re-plated on glass coverslips as 3D clusters. Following 10 days of culture, cardiomyocytes were ready for downstream experimentation. For certain protein studies, iPSC-CMs were treated with chloroquine (cat. C6628, Sigma) at 20 µM for 2 days, brefeldin A (cat. B-8500, LC Laboratories) at 500 ng/mL for 2 days, or cultured in 100 mM sucrose for 2-weeks.
Engineered Cardiac Tissue Preparation and Functional Testing— Differentiated cardiomyocytes were dissociated from T75 flasks and used to prepare fibrin-based ECTs with a protocol adapted from (23,24). Prior to ECT formation, one million cardiomyocytes from the single cell suspension were fixed in 2% PFA and processed for cTnT (Cardiac Isoform Ab-1, Thermo) flow cytometry to determine CM purity. Only ECT data from CM preparations that were >80% cTnT+ were included in contractile analysis. The remaining CM suspension was cultured for 3 hours on a rotating platform at 45 RPM in a 10 cm petri dish in ECT medium. The composition of ECT medium is described in (25) as “mouse media”. Cell clumps from rotational culture were mixed with scaffold reagents in the following ratios: 1.2 million cells: 5 mg fibrinogen: 0.5 units of thrombin in 200 µL total volume of ECT medium. Each ECT was prepared from 200 µL of the mixture pipetted into 20 mm x 3 mm cylindrical molds of the Flexcell Tissue Train silicone membrane culture plate (Flexcell International) according to methods detailed in (25). Gelatinized ECTs were then maintained for 2 weeks in ECT medium containing tranexamic acid (Sigma) at 400 µM and aprotinin (Sigma) at 33 µg/mL. A subset of ECTs were externally paced with the C-pace apparatus (IonOptics) for 1 week at 2.5 Hz after an initial 1 week of culture before being harvested for functional studies. Force measurements were made in a model 801B small intact fiber test apparatus (Aurora Scientific) as previously described (25) while being perfused with Krebs-Henseleit buffer and externally paced. A stationary arm and force transducer (model 403A, Aurora Scientific), to which the ECT is attached at both ends with sutures, were controlled by a micrometer for stretch length measurements. Twitch recordings from which contractile kinetics were measured were obtained at the length of maximum isometric force.

Protein Isolation for Western Blotting — Protein from confluent cardiomyocyte cultures were harvested by directly adding RIPA buffer to each well after being washed once with PBS (no Ca2+ or Mg2+). 1-well per 12-well plate was harvested for measurements of cardiomyocyte purity. For protein studies only preparations >90% cTnT+ were used. Cells were transferred to a microfuge tube, sonicated, and then subjected to a methanol:chloroform:water biphasic extraction according to (26). Protein pellets were redissolved in 5% SDS solution and stored at -80°C until analysis. Samples were boiled in 5% β-ME before electrophoresis. SDS-PAGE was performed with Bio-Rad 4-15% criterion precast gels for 42 minutes at 201 V, and proteins were transferred in a Criterion Blotter with Plate Electrodes to Immobilon-FL PVDF membranes (Millipore) for 18 hours 11 minutes at 11V in Towbin’s buffer. Blotting was performed with the following primary antibodies: anti-LC3 (Cat. No. NB100-2220, Novus Biologicals), LAMP1 (clone H4A3, Developmental Studies Hybridoma Bank-DSHB), LAMP2 (clone H4B4, DSHB), p62 (Cat. No. ab56416, abcam), mono- and poly-ubiquitin (Cat. No. BML-PW8810, Enzo Life Sciences, clone FK2) and GAPDH (Cat. No. G9545, Sigma). Washes were performed in TBS with 0.05% Tween-20 (TBST) and blocking with 5% goat serum. For dystroglycan analysis, WGA-agarose beads (Vector Labs cat. AL-1023) were incubated with iPSC-CM total protein lysates for glycoprotein enrichment. The WGA-bound glycoprotein fraction was then processed for immunodetection of α- and β- dystroglycan and laminin-binding capacity according to methods in (24).

Proteins were concentrated via biphasic extraction, separated, and transferred to PVDF as described before. The following antibodies were used to probe the glycoprotein fraction: βDG (clone 43DAG1/8D5, abcam), anti-aDG (clone IIH6/c4, DSHB), anti-αDG (clone VIA4-1, DSHB). The Laminin overlay assay was performed as described in (27) with the following reagents: EHS Laminin (Sigma, cat. L2020), anti-Laminin (Cat. L9393, Sigma). Secondary antibodies were all Alexafluor conjugated with fluorescent signals detected with the BioRad Chemidoc system.

2-D Western Blotting - Electrophoresis in 2 dimensions began with the carrier ampholine method of isoelectric focusing (IEF) according to (28,29) within a glass tube containing 4% polyacrylamide and 2% of pH 3-10 isodal servalytes (Serva, Heidelberg, Germany) in a 9M urea buffer. IEF was performed for 9600 volt-hrs with WGA glycoprotein extracts from 4 million cells boiled in 5% SDS/5%βME solution and then mixed with 9M Urea/2M Thiourea in a 1:1
ratio before being applied to the acidic end of the tube. Following IEF, the tubes were equilibrated for 5 minutes in 10% glycerol, 50 mM DTT, 2.3% SDS and 0.0625 M tris at pH 6.8 and then boiled for 5 minutes in a water bath. The tube gels were sealed on a stacking gel overlaying a 7% acrylamide separating gel. SDS-PAGE occurred for 4 hrs at 15 mA. The gel was then transferred to PVDF membrane for 18 hours and 1 minute at 22 V with a criterion tank blotter in Towbin’s buffer. Western blotting was then identical to that described for the 1-D method.

Immunocytochemistry/immunohistochemistry – Cardiomyocytes on glass coverslips were fixed in 10% Neutral Buffered Formalin for 15 minutes at room temperature, washed with TBS, permeabilized in 20 µg/mL Digitonin for 10 minutes, blocked in 5% goat serum, and probed with the following antibodies in addition to the ones used for immunoblotting: Golgin-97 (clone CDF4, Novex), and GM130 (clone EP892Y, Abcam). For ECT histology, the tissues were immersed in Dent’s fixative overnight at 4°C, embedded in paraffin and sectioned at 8 µM thickness. Once on glass slides and rehydrated the sections were stained with H&E or cTnT. All nuclei were detected with DAPI.

Electron Microscopy – Cardiomyocyte clusters were fixed in 2.5% paraformaldehyde/3% gluteraldehyde in 0.1M cacodylate buffer overnight at 4°C, post-fixed in 1% osmium tetroxide, dehydrated in ethanol gradient and embedded in Durcapan (Fluka), sectioned at 60 nm and stained with Lead Citrate and Uranium Acetate. All images were taken on a Phillips CM120 TEM.

**N-linked glycan identification by MALDI-TOF-MS** – 10 million iPSC-CMs per cell line were subjected to chloroform:methanol:water lipid extraction three times and then the pellets were washed/precipitated with cold acetone:water three times. A weighed amount of high protein powder from each sample was digested with trypsin/chymotrypsin in Tris-HCl buffer overnight. After protease digestion, each sample was passed through a C18 sep pak cartridge, washed with 5% acetic acid and the glyccopeptides were eluted with a blend of isopropanol in 5% acetic acid.

The glycopeptides were treated with PNGaseF to release the N-linked glycans and each of the digests was passed through a C18 sep pak cartridge to recover the N-glycans. The N-glycans fractions were subsequently lyophilized and permethylated for structural characterization by mass spectrometry (30). Each dried eluate was dissolved with dimethylsulfoxide and methylated with NaOH and methyl iodide. The reaction was quenched with water and per-O-methylated carbohydrates were extracted with methylene chloride and dried under N₂. The permethylated glycans were dissolved with methanol and crystallized with α-dihydroxybenzoic acid (DHBA, 20 mg/mL in 50% methanol:water) matrix. Analysis of glycans present in the samples was performed in the positive ion mode by MALDI-TOF/TOF-MS using AB SCIEX TOF/TOF 5800 (Applied Biosystem MDS Analytical Technologies). The above N-linked glycan mass spectrometry studies were performed by the Complex Carbohydrate Research Center Analytical Services at the University of Georgia, Athens, Georgia.

**RESULTS**

**Reprogramming Pompe disease fibroblasts into iPSC cells** – Skin fibroblasts from 2 unrelated infantile-onset Pompe patients, designated Pompe 1 and Pompe 2, were reprogrammed into iPS cells as previously described (19). In addition, skin fibroblasts from the healthy mother of the Pompe 1 patient, referred to as Control 1, were also reprogrammed. Consistent with the cells being reprogrammed to a pluripotent state, nuclear immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotency-related transcription factor OCT4 was present as was cell surface immunolabeling for the pluripotencyspecific embryonic antigen 4 (SSEA4) (Fig. 1A). The cell line karyotypes were confirmed as normal 46XX in Pompe 1 and Control 1 and 46XY in Pompe 2 (Fig. 1B). The new cell lines were tested for pluripotency using a teratoma assay in immunocompromised mice. As shown in Fig. 1C, formation of all three embryoic germ-layers occurred for each of the new iPSC cell lines. The other human iPSC cell line studied in this work, called Control 2, was previously reprogrammed from lung fibroblasts and characterized (31). Table 2 summarizes the source and properties of the cell lines used in this study.

Pompe iPSC-cells have disease-causing acid α-
glucosidase mutations resulting in undetectable mature protein and enzymatic activity — The mutations in GAA in the original Pompe fibroblasts were evaluated in the corresponding iPS cell lines. The Pompe 1 cell line has a homozygous exon 18 deletion of approximately 550 bp in GAA as demonstrated by the PCR product in Fig. 2A of the genomic DNA region surrounding exon 18. The Control 1 iPS cell line, originating from skin fibroblasts of the mother of Pompe 1, is heterozygous for the exon 18 deletion in the GAA gene. In comparison, the Control 2 iPS cell line is homozygous wild-type for exon 18 of GAA.

The Pompe 2 line is a null compound heterozygote at the GAA locus with one allele containing a deletion of a T nucleotide at cDNA position 1441 (Fig. 2B), resulting in a frameshift and pre-mature stop codon 39 amino acids downstream. The other allele has a G to A transition at position 2237 (Fig. 2B), producing a UAG stop codon at that location. For comparison, the wild-type sequence from Control 2’s genomic DNA are shown.

To test the impact of the Pompe mutations on the expression of GAA protein, western blotting of iPS cell protein lysates with an anti-GAA antibody revealed the active lysosomal 70 and 76 kDa forms of GAA in control but not Pompe lines (Fig. 2C). No significant immunoreactivity for GAA was found from the Pompe 2 sample, but the Pompe 1 sample demonstrated the GAA precursor around a molecular weight of 110 kDa like the controls (32). This is consistent with a previous report that the deletion of exon 18 allows for a precursor peptide to be translated, but not processed into an active form (33).

To ascertain whether the mutations in the Pompe cell lines affects GAA function, protein lysates from iPS cells were assayed for GAA activity via incubation with the substrate 4-methylumbelliferyl-α-D-glucoside (4-MUG) at a pH of 4. Hydrolysis of this substrate releases the fluorophore 4-methylumbelliferone (4-MU) and free glucose. Both Pompe lines had no detectable GAA activity compared to the control lines that demonstrated activity in the expected range (Fig. 2D). The GAA activity of Control 1 was approximately ½ that of Control 2, suggestive of a gene dosage effect since Control 1 has one functional GAA allele while Control 2 has both copies active. As an assay control, 4-MUG was incubated at pH 7 to measure neutral-α-glucosidase (NGA) activity. Equivalent NGA activity was measured in all lines, confirming the catalytic deficit in Pompe disease is due to the hydrolysis of the α1-4 glucosidic bond at the typical acidic pH of the lysosome.

Pompe iPSC-derived cardiomyocytes have pathognomonic glycogen-filled lysosomes — A feature found in striated muscle from patients with Pompe disease is lysosomal glycogen accumulation (34). We examined the ultrastructure of Control and Pompe iPSC-CMs under standard culture conditions by using electron microscopy. The cytoplasm of both Pompe and Control iPS-CMs showed abundant glycogen β-particles in Fig. 3A, a feature found in embryonic cardiomyocytes (35). In order to resolve lysosomal from cytoplasmic glycogen, the iPS-CMs were cultured in media without glucose overnight. Ultrastructure of Control 1 iPS-CMs is identified with electron dense material without evident glycogen. Likewise, the Control 2 and Pompe 2 iPS-CMs showed a comparable distinction in lysosomal glycogen accumulation (data not shown).

Contractile function of engineered cardiac tissue produced with Control and Pompe iPS-CMs— Given the limited understanding of cardiac contractile function in Pompe disease in the setting of profound cardiac hypertrophy, we produced engineered cardiac tissue (ECT) with iPS-CMs to assess contractile performance. Following 2 weeks of culture, the ECTs remodeled into linear strips of spontaneously contracting tissue (Fig. 4A, movie 1) that were cellularized (Fig. 4B) with cardiomyocytes located circumferentially as visualized with cTnT immunofluorescence (Fig. 4C). Electron micrographs of Control and Pompe ECTs displayed elongated nuclei and longitudinally oriented myofilaments (Fig. 4D and 4E). However, only in the Pompe ECT were glycogen filled lysosomes evident.

ECTs were stimulated at 2.5 Hz and
isometric force generation was measured. Pompe 1 ECTs demonstrated significantly accelerated kinetics of contraction compared to the other lines tested (Fig. 5A, Table 3). However, we noted differences in the intrinsic rates at which the ECTs contracted during the 2 weeks of culture during ECT maturation. Pompe 1 ECTs had an intrinsic rate (2.2 Hz) that was at least double the intrinsic rate of the other lines studied (Table 3). Therefore, we conducted another series of experiments in which all ECTs were conditioned by pacing at 2.5 Hz for one week in culture prior to force measurements to provide a uniform rate of contraction. Pacing for one week accelerated the contraction kinetics for the three lines that had slower intrinsic rates to a greater extent than for Pompe 1 ECTs that had a fast intrinsic rate (Fig. 5B). For the conditioned ECTs, the normalized force \( \frac{F}{F_{\max}} \) and the first derivative \( \frac{dF}{dt} / F_{\max} \) vs. time relationships (Table 3) showed no consistent differences between ECTs prepared from the 2 Control and 2 Pompe cell lines (Fig. 5C and 5D, Table 3).

**Macro-autophagic function is preserved in Pompe iPSC-CMs**—Recent studies in Pompe skeletal muscle have implicated impaired autophagic flux as a major contributor to the pathogenesis of myofiber destruction (8). However, the role of autophagic dysfunction in the myocardium of infantile-onset Pompe patients is unknown (14). To examine autophagosomal turnover in the Pompe iPSC-CM model, we forced autophagosome accumulation by blocking lysosomal acidification with chloroquine (CQ). Removal of CQ from the media re-establishes lysosomal function and autophagosomal clearance (36). To detect the autophagosomal system, we examined the microtubule-associated protein light chain 3 (LC3) which is synthesized in a soluble cytoplasmic form (LC3-I) and upon induction of autophagy, becomes lipidated and anchors in the autophagosome membrane as LC3-II. Immunofluorescence studies demonstrate LC3 fluorescence on day 0 (D0) is diffuse through the cytoplasm, highlighting the LC3I form; however, following CQ exposure the fluorescence becomes more intense and punctate, representing conversion to the membrane localized LC3II form (Fig. 6A). These results demonstrate that the treatment with CQ cause autophagosomal accumulation in the iPSC-CMs.

Western blots with anti-LC3 were evaluated for iPSC-CMs during CQ treatment and recovery along with anti-GAPDH loading controls (Fig. 6B). Densitometric ratios of LC3II/GAPDH show that all lines experienced a rise in LC3II following 48 hours of 20 µM CQ treatment (Fig 6C); the rise was between 15-20 fold above baseline ratios. At day 1 of recovery (R1), all lines showed a decline in LC3II, except for Pompe 2 iPSC-CMs, which consistently demonstrated a rise in LC3II at R1. By 8 days of recovery (R8), all Control and Pompe iPSC-CMs were able to reduce their LC3II burden towards baseline (D0) levels. Therefore, a CQ-based autophagic challenge did not reveal persistent autophagosomal (LC3II) accumulation or a slower autophagic flux in Pompe iPSC-CMs compared to the Control iPSC-CMs. Secondary markers of autophagic dysfunction, such as the autophagy adaptor protein p62 and poly-ubiquitylated proteins, were not significantly different in Pompe versus Control iPSC-CMs (fig 6D, E).

The lysosomal system also changes dramatically when exposed to CQ, visualized in Fig. 7A with anti-lysosomal associated membrane protein 1 (LAMP1) marking the lysosomes (37). Before CQ treatment, LAMP1 labeling is punctate in Control iPSC-CMs, but outlines larger lysosomal structures in Pompe cells. In response to CQ, both Pompe and Control cells experience an expansion of the lysosomal population, both in quantity and/or size.

The LAMP1 immunoblots in Fig. 7B show an increase in intensity following CQ treatment for the Control iPSC-CMs, but in the Pompe iPSC-CMs an unexpected change in electrophoretic mobility of LAMP1 was observed. LAMP1-SDS complexes from control cells have an apparent molecular weight of 100 kDa, while LAMP1 in the Pompe lines present as a smear with prominent bands below 100 kDa. Following CQ treatment, the intensity of the lower mobility LAMP1 band in the Pompe lines increased concomitant with diminishing amounts of the higher mobility forms. The mobility of LAMP1 in the Control lines remains the same upon CQ exposure. We also investigated the immunoblotting patterns of LAMP2 (Fig. 7C), which co-exists with LAMP1 as a glycoprotein on the lysosomal membrane. Like LAMP1, LAMP2 presents as a single band in the Control lines, but
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as multiple bands of higher mobility in the Pompe lines. Also like LAMP1, Control lines experience an increase in LAMP2 intensity with no change in mobility as a result of CQ exposure while Pompe cells demonstrate a reduction in LAMP2 SDS-complex mobility in response to CQ.

Lyposomal membrane proteins are hypoglycosylated in Pompe iPSC-CMs – We decided to investigate the cause for the differences in mobilities of the LAMPs in the Pompe iPSC-CMs by focusing on glycosylation since both of these proteins are heavily glycosylated by traditional N- and O-linked ER-to-golgi glycan biosynthesis pathways. In addition, several of the N-linked adducts are modified by long poly-N-acetyllactosamines added in the golgi apparatus (38-41). To test the hypothesis that a deficit in golgi-based glycosylation is the source of lower molecular weight LAMP species, iPSC-CMs were exposed to brefeldin A (BFA), a fungal metabolite that causes collapse of the golgi-stacks disrupting golgi-based glycosylation (42). Immunolabeling of Control and Pompe iPSC-CMs for the cis-golgi marker GM130 and trans-golgi marker golgin-97 (Fig. 8A) confirmed that BFA treatment dismantled the golgi stack cis-to-trans orientation moving outwards from the nucleus. As a result of golgi disruption, Control iPSC-CMs harbored the higher mobility forms of LAMP1 and LAMP2 normally present in the Pompe group (Fig. 8B). Similarly, the Pompe iPSC-CMs treated with BFA show an increase in the intensity of the higher mobility LAMPs at the cost of the lower mobility species. Therefore, we were able to reproduce in the Control lines the immunoblotting pattern of the LAMPs unique to the Pompe lines by interfering with the Control iPSC-CM golgi’s ability to glycosylate proteins. Moreover, the Pompe disease-specific LAMP patterns were exaggerated in the Pompe iPSC-CMs through golgi disruption.

Since Pompe iPSC-CMs had no overt golgi structural pathology, we next tested if the hypoglycosylation of the LAMP proteins was a general response to lysosomal expansion or specific to lysosomal glycogen accumulation occurring in Pompe disease. We cultured the iPSC-CMs in 100 mM sucrose to induce a “sucrosome” storage disorder (43). In high sucrose concentrations, cells lacking invertase activity gradually accumulate cytoplasmic sucrose, which enters the endosomal/autophagic system and is ultimately trapped in the lysosome. Control 2 iPSC-CMs demonstrated lysosomal expansion marked by LAMP1 due to sucrose uptake (Fig. 8C). However, immunoblot detection of LAMP1 and LAMP2 following sucrose culture (Fig. 8B) revealed no changes in species mobility in the Control 1 and Control 2 iPSC-CMs, suggesting that the higher mobility LAMPs from Pompe cells is not due simply to lysosomal expansion from accumulation of a nonmetabolizable carbohydrate. Sucrose treatment did increase the lower mobility LAMP1 and LAMP2 band intensities in the Pompe lines, indicating that the new LAMPs being synthesized were undergoing correct golgi-glycosylation.

In contrast to the different mobilities of LAMP1 and LAMP2 in Pompe and Control iPSC-CMs, no differences in LAMP protein mobilities were observed in the original skin fibroblasts or undifferentiated iPS cells (Fig. 8D). The cell type dependence of LAMP hypoglycosylation has implications for understanding the tissue-specific manifestation of Pompe disease pathology.

To further characterize the deficit of glycosylation, protein lysates were tested for endoglycosidase sensitivity. Treatment of lysates from iPSC-CMs with Endoglycosidase F (EndoF), which cleaves all N-linked glycans at their asparagine, produces a de-N-glycosylated LAMP2 protein of equal molecular weight in Pompe and Control cells (Fig. 9A). Protein lysates were also subject to endoglycosidase H (EndoH) treatment to determine the extent of golgi glycan-processing. EndoH only cleaves N-glycans if they have not been acted upon by the golgi enzyme α-mannosidase II (αManII), the committing reaction to downstream complex N-linked glycan synthesis (44). Control 2 and Pompe 2 lines showed a subtle increase in LAMP2 mobility upon EndoH treatment, implying that a sub-population of N-linked glycans are not processed by αManII (Fig. 9A). Similar EndoH sensitivity between Control 2 and Pompe 2 LAMP2 suggests the deficit in golgi processing is not from lack of entry into the golgi or the inaction of golgi-mannosidases, but by reduced golgi-glycotransferase activity responsible for post-αManII complex glycan extension. Results from LAMP1 endoglycosidase treatment and from the Control 1 and Pompe 1 lines were the same.

The LAMPs have a significant number of
sialic acid sugars added in the golgi (45). We hypothesized that incomplete golgi-based glycosylation in Pompe iPSC-CMs that affects apparent molecular weight would also change the pI due to incomplete addition of charged sugars such as the sialic acids. To evaluate for this, we performed isoelectric focusing (IEF) in 2-D polyacrylamide gel electrophoresis followed by immunodetection of LAMP2.

Enrichment of glycoproteins via wheat germ agglutinin (WGA) pulldown was necessary to immunodetect the LAMPs following 2D separation and membrane transfer. The WGA method increased the amount of LAMP2 protein-to-total protein ratio and captured the higher mobility species observed in Pompe iPSC-CMs (Fig. 9B). The flow-through contained an undetectable amount of LAMP2 (Fig. 9C). Therefore, data from the 2D-bLOTS are not being confounded by the purification method employed.

Isoelectric separation revealed LAMP2 detection at pH 4.6 from Control 1 & 2 cells, at pH 5.6 from the Pompe 1 cells and as a distribution between pH 5.0-6.4 in Pompe 2 cells (Fig. 9D). Treatment of WGA-enriched glycoprotein with sialidase, an exoglycosidase with specificity for terminal sialic acid carbohydrates, produced an alkaline pI shift in Control 1 LAMP2 to pH 5.3-6.1, but had little to no effect on the pI of Pompe 1 LAMP2 (Fig. 9E). Therefore, The pI studies are consistent with a reduction of acidic sugar (sialic acid) addition to LAMP2 in Pompe iPSC-CMs.

Pompe iPSC-CMs have a global deficiency in N-linked glycan synthesis For an assessment of total N-linked glycans, MALDI-TOF-MS was performed on purified N-linked glycans released by EndoF treatment of 10 million Control or Pompe iPSC-CMs per sample. Mass spectra of Control 1 and Pompe 1 (Fig. 10) and Control 2 and Pompe 2 (Fig. 11) N-linked glycans demonstrate the presence of all high-mannose structures in all cell lines (table 4). A major peak at m/z = 1345.6 corresponding to a fucosylated chitobiose trimannose structure (Fuc)(NacGln)2(Man)3 was present exclusively in the Pompe spectra (Fig. 10B, 11B). All complex-type N-linked glycans have a tri-mannose core produced by the αManII/IX class of golgi-glycosidases before branching and extension into complex glycans (46). The unmodified tri-mannose core was not detected in the control spectra (Fig. 10A, 11A). Both Pompe and Control cells have bi-antennary structures with N-acetyl-glucosamine, galactose, and sialic acids. However, Pompe cells are deficient in the higher complexity bi-antennary and multi-antennary glycans (table 4).

Golgi-based glycosylation and laminin binding capability of α-dystroglycan is preserved in Pompe iPSC-CMs From the finding that the deficit of Pompe LAMP glycosylation was in the golgi, we hypothesized that α-dystroglycan (αDG) is also hypoglycosylated in Pompe iPSC-CMs, since αDG undergoes O-linked glycosylation via the like-acetylglucosaminyltransferase (LARGE) located in the golgi (47). The disaccharide polymer (xylose-glucuronic acid), (48) added by LARGE function as the binding domain for laminin that when lacking causes a class of muscular dystrophies called the dystroglycanopathies (27,48,49). Since Pompe disease shares a similar clinical presentation with dystroglycanopathies (50), we tested the hypothesis that a loss of laminin binding by αDG contributes to Pompe cardiomyopathy.

Antibody clones IIH6c4 and VIA4-1 that only recognize the LARGE processed form of αDG (51,52) were used in quantitative immunoblot assays of WGA-enriched glycoprotein extracts from Pompe and control iPSC-CMs. The glycosylated αDG (VIA4-1, IIH6c4) to βDG band density ratios were the same in Pompe and Control iPSC-CMs (Fig. 11A and 11C), arguing against a deficit of αDG glycosylation. To confirm that glycosylated αDG in Pompe iPSC-CMs was functionally able to bind laminin, we performed a laminin overlay assay. Both control and Pompe iPSC-CMs exhibited comparable αDG specific laminin binding (Fig. 11B and 11C). In addition, following 2 days of culture in laminin containing media both Control and Pompe iPSC-CMs showed a dramatic increase in surface laminin fluorescence (Fig. 11D and 11E). The co-localization of IIH6c4 and laminin fluorescence is in support of αDG mediated laminin binding (53,54).

DISCUSSION
This study establishes an iPSC-CM model of infantile-onset Pompe cardiomyopathy that manifests pathognomonic features – undetectable GAA activity and the presence of glycogen-filled....
lysosomes. Characterization of the Pompe iPSC-CMs did not reveal clear abnormalities in contractile function or the autophagic process; however, we unexpectedly found changes in the electrophoretic mobility of LAMPs in Pompe compared to controls iPSC-CMs. These changes were due to golgi-based hypoglycosylation of the LAMPs, which typically include poly-N-acetyllactosamine and sialic acid. This study unmasks a previously unknown abnormality in protein processing in Pompe iPSC-CMs with features of hypoglycosylation similar to other congenital disorders of glycosylation (CDG) that also manifest hypertrophic cardiomyopathy (17, 55-57).

Patients with Pompe cardiomyopathy present with marked cardiac hypertrophy, which is occasionally diagnosed in utero. Echocardiography studies have demonstrated that systolic function is preserved despite profound hypertrophy for the first 5-6 months of life (2, 5), but diastolic function (relaxation) of the heart is impaired (58). By generating ECTs from iPSC-CMs, we sought to examine the early Pompe cardiomyopathy. Once we controlled for intrinsic rate by pacing all constructs at a fixed rate of 2.5 Hz for one week, no clear differences in contractile performance between the two control lines and two Pompe lines were observed. The fact that we did not find delayed relaxation in the Pompe ECTs as seen clinically may indicate that those changes are secondary to macroscopic remodeling of the heart rather than being intrinsic to the cardiomyocytes. The lack of significant contractile abnormalities suggests that contractile dysfunction is less likely to be the primary stimulus of the hypertrophic phenotype in Pompe cardiomyopathy.

Autophagic dysfunction has been recognized as an important feature in human Pompe skeletal myopathy (9). Elevation of the autophagosomal marker LC3II in Pompe iPSC-CMs was not observed under standard culture conditions or after a CQ-induced autophagic challenge. The auxiliary markers of insufficient autophagy, p62 and conjugated-ubiquitin, were also not elevated in Pompe iPSC-CMs. Thus, results from our model argue that autophagic dysfunction is not central to the early Pompe cardiomyopathy in humans. The Pompe mouse model also lacks autophagosomal accumulation in heart tissue (59) despite it being observed in skeletal muscle.

Abnormalities in glycosylation of LAMPs in Pompe iPSC-CMs were suggested by higher mobility LAMP species on immunoblots. In western blots of Pompe mouse heart tissue, LAMP2 presented with higher mobility forms not detected in control hearts (60). Other CDGs have also been identified by variations in SDS-complex mobility of LAMP2. For example, hypoglycosylated LAMP2 has been observed in Cohen’s syndrome which is caused by mutations in a protein necessary for normal golgi structure (61). In CDGs due to mutations in the conserved oligomeric Golgi (COG) complex (62) or TMEM165 (63), loss of golgi organization has been associated with LAMP2 hypoglycosylation.

Hyposialylation is a common, diagnostic feature of CDG (64-66). The LAMPs undergo extensive sialylation – 36.4 and 23.6 moles of sialic acid are added per mole LAMP1 or LAMP2, respectively (38). The higher pIs and limited sialidase sensitivity of LAMP2 in Pompe iPSC-CMs compared to controls is consistent with a deficiency of sialic acid modification.

A key similarity amongst many of the CDGs is either a disruption of golgi structure, or the loss of a glycoprocessing protein’s activity (67, 68). In our work, we did not identify any obvious disruption in golgi stack structure nor does GAA have a known role in protein glycosylation. We speculate that a link between insufficient GAA activity and hypoglycosylation is secondary to changes in glycogen metabolism. A recent publication using a mouse model of Pompe Disease demonstrated dramatically increased activity of glycogen synthase concomitant with reduced glycogen phosphorylase activity, particularly in the heart (69). Since both glycogen synthesis and the nucleotide sugar synthesis pathways use UTP and glucose-6-phosphate as substrates, these reactants may be disproportionately consumed in glycogen synthesis resulting in limited nucleotide sugar concentrations for golgi glycotransferase reactions. Based on this reasoning, the ability for the acidotrope CQ to reverse the hypoglycosylation could arise from the neutralization of the cis-to-trans golgi pH gradient, thereby slowing transport of proteins through the golgi (70), giving the LAMPs more time to be
glycosylated in the environment of low substrate concentrations (40). Our finding that lysosomal sucrose accumulation had no effect on LAMP mobility in Control iPSC-CMs is also supportive of glycogen accumulation being essential to the observed hypoglycosylation. In addition, Glycogen storage disease (GSD) type 1b has also been identified as a CDG (71), giving precedence to a relationship between abnormal glycogen metabolism in a glycogen storage disorder and deficits in golgi-glycosylation (72).

Limited nucleotide sugar concentrations in the Pompe iPSC-CM golgi can also explain the mass spectrometry results. The enzymes N-acetylglucosaminyl-transferase (GnT) II, III, and V are required to make bi-, bisecting bi-, and multi-antennary N-linked glycans, respectively. The $K_m$ for their common substrate, UDP-N-acetylglucosamine (GlnNac), is 18 $\mu$M (73), 420 $\mu$M (74), and 4 mM (75) for GnT II, III, and V, respectively. Therefore, the equal presence of biantennary structures in Pompe and Control iPSC-CMs, but limited bisecting-biantennary and absent multi-antennary structures in Pompe cells may be caused by the increasing UDP-GlnNac concentration dependance for the necessary GnT activity.

The tri- and tetra-antennary structures are the preferred substrates for poly-N-acetyllactosamine extensions that are abundant on the LAMPS (76). From previous pulse-chase experiments (38), lactosaminoglycan addition was responsible for an ≈25 kDa rise in LAMP molecular weight following golgi processing. The 25 kDa molecular weight range for LAMPS from Pompe cells may be due to a variable lack of lactosaminoglycan extension. The absence of these extensions may originate from a deficiency in early glycan branching.

The fucosylated chitobiose tri-mannose glycan detected as a major peak in the Pompe mass spectra suggests that N-linked glycans are being prepared for glycan branching and extension by a $\alpha$ManII/IIX-type glycosidase (46), however, these glycans do not experience further processing. There is debate whether the unmodified trimannose core can be produced by $\alpha$ManII/IIX (77), however, $(\text{Fuc})_3(\text{NacGln})_3(\text{Man})_3$ at $m/z = 1345.6$ has been observed in MALDI-TOF from other cells of mammalian origin (78). Whether the tri-mannose glycan is an indication of deficient complex glycan synthesis or a purposeful final product requires investigation.

Given the known pathology of hypoglycosylated $\alpha$DG resulting in a loss of laminin binding in a subset of muscular dystrophies which can have associated cardiomyopathies (79,80), we examined the glycosylation of $\alpha$DG specifically. We did not detect differences in $\alpha$DG glycosylation or laminin binding between Pompe and Control iPSC-CMs, suggesting that this specific glycosylation pathway is intact in Pompe disease and unlikely to be central to the pathophysiology.

CDGs are increasingly associated with the presence of a cardiomyopathy (17,81). Mechanisms linking the variety of glycosylation deficits to cardiac pathology are poorly understood; however, several mechanisms merit future consideration. For example, the lack of sialylation of the sphingolipids on the cardiomyocyte plasma membrane has been shown to increase Ca$^{2+}$ permeability (82,83). Higher intracellular Ca$^{2+}$ concentrations are a known trigger for hypertrophic signaling (84). Altered glycosylation of a variety of ion channel proteins expressed in the heart can change the functional properties of the channels (85-88). Thus, changes in key ionic currents could increase intracellular Ca$^{2+}$ levels inducing pathological hypertrophy.

In conclusion, the iPSC-CM model of Pompe disease was used to identify a golgi-based glycosylation deficit potentially central to Pompe cardiac pathophysiology that gives reason to pursue therapeutic avenues aimed at restoring normal glycoprocessing. Future studies will be needed to define how the loss of lysosomal glycogen degradation affects glycosylation and how glycosylation changes are related to the cardiomyopathy. Pompe iPS cell models will provide access to various human cell types relevant for further investigation of the disease and its treatment.
REFERENCES


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FOOTNOTES

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2 The abbreviations used are: iPSC cells, induced pluripotent stem cells; iPSC-CMs, induced pluripotent stem cell derived cardiomyocytes; LC3, light chain 3; GAA, acid α-glucosidase; NGA, neutral α-glucosidase; ECT, engineered cardiac tissue; CQ, chloroquine; BFA, Brefeldin A; LAMP1, lysosomal associated membrane protein 1; LAMP2, lysosomal associated membrane protein 2; WGA, wheat germ agglutinin; MALDI-TOF-MS, Matrix assisted laser desorption ionization time-of-flight mass spectrometry; LARGE, like-acetylglucosaminyltransferase α-DG; α-dystroglycan; β-DG, β-dystroglycan; CDG, congenital disorder of glycosylation; LVEF: left ventricular ejection fraction.

TABLE LEGENDS

Table 1 Primers for GAA genotyping Genomic DNA from iPS cells were amplified with the tabulated primer pairs and the GoTaq Green (Promega) polymerase mix. The DNA products were analyzed as described in the methods.

Table 2 IPS-cell line nomenclature, GAA genotype, and phenotype del ex18: deletion of exon 18; WT: wild-type; 1441delT: deletion of a T nucleotide at GAA cDNA position 1441; 2237G>A: G to A transition at GAA cDNA position 2237.

Table 3 Comparison of kinetic parameters and maximum force measured at a pacing frequency of 2.5 Hz between ECTs contracting at their unpaced rate or paced at 2.5 Hz for 1 week prior to testing. Time to peak: time from external pacing voltage initiating the contraction to peak force generation. Time to 50% force relaxation: Time from maximum force to 50% maximum force during relaxation. The Ns for each group refer to the number of ECTs tested. Errors are ± S.E.

Table 4 Structures of N-linked glycans from Pompe and Control iPSC-CMs identified by MALDI-TOF-MS The mass/charge (M+H+) of permethylated glycans were matched to the permethylated molecular weights of the formula (Hex)n(HexNac)m(DeoxyHex)n(NeuAc)h where Hex = Hexose, Nac = N-acetyl, NeuAc = Neuraminic acids (sialic acids). Structural formulas were assigned based on known N-linked glycan structures for a given molecular formula. The glycans are arranged from top-to-bottom based on the complexity of golgi processing with the simplest glycans of the high-mannose variety at the top to the tri- and tetra-antennary structures at the bottom.
MOOVIE LEGEND

Movie 1 ECT from the Control 2 line contracting in the perfusion chamber. The ECT is attached to the stationary arm with surgical sutures. The free end will then be attached to the force transducer during testing.

FIGURE LEGENDS

Fig. 1. Characterization of iPS cells reprogrammed from patient dermal fibroblasts A. Immunofluorescence of iPS cell cultures probed with anti-OCT4 (nuclear) and anti-SSEA4 (plasma membrane), markers of pluripotency. Scale bar = 100 μm for panels and 10 μm for insets. B. Giemsa-band Karyograms from each line. Control 1: 46XX; Pompe 1: 46XX; Pompe 2: 46XY. C. H&E stained sections of iPS cell teratomas. Examples of endoderm, mesoderm, and ectoderm are represented from each reprogrammed line. Endoderm – Control 1: Hepatoid cells; Pompe 1: Primitive gut epithelium; Pompe 2: Respiratory epithelium. Mesoderm – Control 1: Cartilage; Pompe 1: Smooth muscle; Pompe 2: Cartilage. Ectoderm – Control 1, Pompe 1 & Pompe 2: Retinal Pigmented Epithelium. Scale bar = 50 μm.

Fig. 2. The acid α-glucosidase genotype and biochemical phenotype in Pompe and Control iPS cell lines. A. PCR products of the genomic DNA region that includes GAA exon 18 from Pompe 1, Control 1, and Control 2 iPS cell lines separated on a 0.8% agarose gel. ex18: exon 18. Del ex18: deletion of exon 18. B. Sequencing chromatograms of PCR products from Pompe 2 iPS cell genomic DNA at locations of the 2 point mutations. Aligned wild-type sequences underneath are from Control 2 iPS cells. Arrows point to the deletion of a T nucleotide in one allele and the G>A transition in the other allele. Regions of interest were PCR amplified and directly sequenced. Nucleotide position numbers refer to the GAA cDNA sequence. C. An immunoblot of iPS cell total protein lysates probed with anti-GAA. The precursor form is approximately 110 kDa and enzymatically active forms are represented by a dark band above and a light band below the 75 kDa marker. Anti-GAPDH was used as a loading control. D. Enzymatic activity assay of total protein lysates from the 4 iPS cell lines for the ability to hydrolyze 4-methylumbelliferyl-α-D-glucoside (4-MUG) into glucose and the fluorophore 4-methylumbelliferone (4-MU) at pH = 4 for lysosomal GAA and at pH = 7 for cytoplasmic neutral α-glucosidase. Activity is measured as nmol of 4-MU released per mg of protein per hour. N = 4 biological replicates (cells taken from different passages) for each line. All error bars are ± S.E.

Fig. 3. Ultrastructure of Pompe and Control iPSC-CMs A. Electron micrographs of Control 1 and Pompe 1 iPSC-CMs cultured in media containing 4.5 g/L glucose. Scale bar = 5μm. B. Electron micrographs of Control 1 and Pompe 1 iPSC-CMs cultured in zero g/L glucose for 24 hours before imaging. Scale bar = 5 μm. C. Expanded regions from panel B. scale bar = 0.5 μm. Legend: N = Nuclei; CG = cytoplasmic glycogen; M = mitochondria; HL = healthy lysosome; LG = lysosomal glycogen; MF: myofilaments; MFx: Myofilaments in cross-section.

Fig. 4. Structural characterization of ECTs produced with Control and Pompe iPSC-CMs A. Photograph of an ECT in perfusion chamber before testing. The left end is tied to a stationary arm and the right end will soon be attached to the force transducer. Scale bar = 1 mm. B. Photomicrograph of an H&E stained longitudinal ECT section post-testing. Scale bar = 50 μm. C. Immunofluorescence image of a sectioned ECT immunolabeled with anti-αTnT to identify cardiomyocyte location. Scale bar = 50 μm. DAPI stains the nuclei blue. D. Representative electron micrographs of a Control ECT. Scale bar = 5 μm. E. An electron micrographs of a Pompe ECT. N = Nuclei; MF = myofilaments; LG = lysosomal glycogen. Scale bar = 5 μm.
Fig. 5. Contractile force and kinetic studies of ECTs produced with Control and Pompe iPSC-CMs. A. Force normalized to maximum force (F/Fmax) vs. time curves for a single contraction at 2.5 Hz from ECTs contracting at their intrinsic rate (unpaced) while in culture for 2 weeks prior to testing. On average 30 contractions are averaged for each ECT. The F/Fmax vs. time curves measured at 2.5 Hz for both Control and Pompe ECTs either allowed to contract at their intrinsic rate for 2 weeks in culture, or paced at 2.5 Hz for 1 week before measurement. The Ns for each cell line in both paced and intrinsic rate groups are given in table 3. C. F/Fmax vs. time curves for a single contraction at 2.5 Hz from ECTs that were paced in culture for 1-week at 2.5Hz. D. First derivative (dF/dt) of the F/Fmax vs. time curves in C. dt = 0.001 sec (?). All error bars are ± S.E.

Fig. 6. Autophagosomal flux in Control and Pompe iPSC-CMs during recovery from CQ mediated lysosomal arrest. A. Immunofluorescence image of LC3 in Control and Pompe iPSC-CMs before and after 2 days of CQ treatment. DAPI stains the nuclei blue. Scale bar = 10 µm. B. LC3 immunoblots of total protein lysates during CQ treatment and recovery with GAPDH controls. LC3I is converted into LC3II upon autophagosomal formation. 3 replicate experiments (CMs differentiated from different iPS cell passages) are shown for each line with one lane representing cells from 1 culture well. D0: Day 0. Cells were harvested immediately before CQ treatment; CQ: Cells exposed to CQ for 2 days before protein collection. R1,R4,R8: Recovery from CQ treatment. CQ was removed from the media and cells were cultured for 1, 4, and 8 days in normal media before harvest. The x-axis is labeled to correspond with immunoblot time points. C. Quantification of LC3II/GAPDH density ratios from panel B relative to the maximum ratio set to 1. Each x-axis mark represents 1 day with the black bar indicating CQ exposure. D. Immunoblots of p62 and total ubiquitinylated protein (mono- and poly-) from Control 1&2 and Pompe 1&2 iPSC-CM total protein. E. Quantification of p62 and conjugated-ubquitin (Conj-Ub) to GAPDH density ratios. Density ratios were averaged from 4 independent Control (2 C1 + 2 C2) and Pompe (2 P1 + 2 P2) samples on 2 separate blots. All error bars are ± S.E.

Fig. 7. Analysis of LAMPs from Control and Pompe iPSC-CMs before and after CQ treatment. A. LAMP1 immunofluorescence in Control and Pompe iPSC-CMs before (D0) and after 2 days of CQ treatment. DAPI stains the nuclei in blue. Scale bar = 10 µm. B. LAMP1 immunoblots from Pompe and Control iPSC-CMs before and after 2 days of CQ treatment. Data is presented in duplicates. C. LAMP2 immunoblots from Pompe and Control iPSC-CMs before and after 2 days of CQ exposure.

Fig. 8. Effect of golgi structural disruption and induction of an artificial lysosomal storage disorder on LAMP1 and LAMP2 from Control and Pompe iPSC-CMs. A. The golgi apparatus stained with cis- and trans-golgi marker GM130 and Golgin-97, respectively, in Control and Pompe iPSC-CMs and after treatment with BFA. Scale bars = 10 µm. B. Immunoblots of LAMP1 and LAMP2 from iPS-CMs with no treatments (No Tx), after 2 days of 500 ng/mL brefeldin A (BFA) treatment, and after 2-weeks of culture in 100 mM sucrose (S). GAPDH functions as the loading control C. Lysosomes stained with LAMP1 before and after 2-weeks of sucrose treatment in Control 2 iPSC-CMs. Scale bar = 10 µm. All nuclei are stained with DAPI in blue. D. Representative LAMP1 and LAMP2 immunodetection from skin fibroblast (FB) and iPS cell total protein lysates.

Fig. 9. Glycosylation analysis of LAMP2 from Control and Pompe iPSC-CMs via endoglycosidase treatment and 2-D glycoprotein separation. A. Endoglycosidase analysis of LAMP2 protein in Control 2 and Pompe 2 iPSC-CMs. Endoglycosidase F (EndoF) cleaves all N-linked glycan chains, producing a de-N-glycosylated peptide. Endoglycosidase H (EndoH) cleaves N-linked glycan chains that have not yet been processed by the α-mannosidase II class of golgi glycosidases. B. Western blots demonstrating LAMP2 enrichment from total iPSC-CM protein by WGA glycoprotein pulldown. LAMP2 was immmodetected from equal amounts of Control 2 and Pompe 2 total cellular protein (input) and WGA-bound glycoprotein (wga). C. Western blot showing efficiency of LAMP2 extraction by WGA from Control 1 iPSC-CM total protein lysate. LAMP2 and GAPDH were immunodetected in the WGA-bound
fraction and the WGA-unbound (flow-through, FT) fraction. **D.** WGA-bound glycoprotein extracts from Control 1&2 and Pompe 1&2 iPSC-CMs separated by pl and molecular weight along the horizontal and vertical axes, respectively, and probed for LAMP2. The pH gradient across the IEF tube was measured by using a surface pH electrode for 3 blank IEF tubes. Panel **D** also includes sialidase treated Control 1 and Pompe 1 glycoprotein prior to 2D separation and immunodetection of LAMP2. * indicates likely background detection.

**Fig. 10. Mass spectra of N-linked glycans from Control 1 and Pompe 1 iPSC-CMs** Mass Spectra obtained from MALDI-TOF-MS of N-linked glycans from Control 1 (panel **A**) and Pompe 1 (panel **B**) iPSC-CMs. Equal (weighed) amounts of total cellular protein per sample were processed for N-linked glycan extraction, permethylation, and mass determination. Individual peaks and background intensities are calibrated to the sample’s maximally intense peak. Regions that contain peaks of relative low intensity have been presented at 4x magnification above the region without magnification.

**Fig. 11 Mass spectra of N-linked glycans from Control 2 and Pompe 2 iPSC-CMs** Mass Spectra obtained from MALDI-TOF-MS of N-linked glycans from Control 2 (panel **A**) and Pompe 2 (panel **B**) iPSC-CMs. See Fig. 10 legend for description.

**Fig. 12. Dystroglycan glycosylation and laminin binding in Control and Pompe iPSC-CMs** **A.** Immunoblots of glycoprotein from both control (C1, C2) and Pompe (P1, P2) iPSC-CMs probed with anti-αDG antibodies IIH6c4 and VIA4-1 that exclusively detect the laminin binding glyco-epitope. Each lane within 1 cell line represents protein from iPSC-CMs differentiated from different iPS cell passages. **B.** Laminin overlay assay to measure laminin binding ability specific to α-DG. Binding of laminin occurs at the molecular weight of αDG recognized by IIH6c4 and VIA4-1. **C.** Quantification of α/β dystroglycan ratios in panel A and laminin binding to βDG ratios in panel D. No significant differences were noticed between control and Pompe groups. **D**&**E.** Laminin and αDG (IIH6c4) immunofluorescence of iPSC-CMs fixed after 2 days of incubation with exogenous laminin (bottom row) compared to normal culture conditions (top row) in Control 2 (**D**) and Pompe 2 (**E**) iPSC-CMs. Scale bar = 20 µm. All nuclei are stained with DAPI in blue.
Table 1

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<td>107±2</td>
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<td>2.5 (9)</td>
<td>187±5</td>
<td>1’166±3</td>
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¹Paced value is significantly different from unpaced value (p <0.05, unpaired t-test with unequal variance)
²P1 is significantly different from all other values (p<0.05, ANOVA*)
³No significant differences were noticed between all comparisons (ANOVA)
⁴Significant differences were found in all comparisons except between the C1,P2 pairing (p<0.05, ANOVA)
⁵Significant differences were only found between the P1,P2 and P1,C1 pairings (p<0.05, ANOVA)
⁶P1 is significantly different from P2 (p<0.05, ANOVA)
⁷P1 is significantly different from C1 (p<0.05, ANOVA)

*The Bonferroni post hoc correction method was used following ANOVA
### Table 4

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</table>
Figure 1
Figure 2

A

B

C

D

Golgi glycosylation defect in Pompe iPSC-cardiomyocytes

Figure 2

A

B

C

D

Golgi glycosylation defect in Pompe iPSC-cardiomyocytes
Figure 3

A. Control 1 vs. Pompe 1 under 4.5 g/L glucose.

B. Control 1 vs. Pompe 1 under 0 g/L glucose.

C. Detailed structures in control and Pompe conditions.
Figure 4
Figure 5

A

B

C

D

Intrinsic Rate ($R_i$) - Paced 2.5 Hz

$C1$ $R_i = 1.1 \text{ Hz}$

$C2$ $R_i = 0.8 \text{ Hz}$

$P1$ $R_i = 2.2 \text{ Hz}$

$P2$ $R_i = 0.6 \text{ Hz}$

$\frac{F}{F_{\text{max}}} = \frac{1}{1}$

$\frac{d(F/F_{\text{max}})}{dt} = 0$
Figure 6

**A** Control 1 vs. Pompe 1
- Control 1 (left)
- Pompe 1 (right)
- 48 hr CQ
- D0, No Tx

**B** kDa
- LC3
- GAPDH
- Control 1
- Control 2
- R1
- R4
- R8

**C** LC3II/GAPDH relative to peak
- Control 1 N=6
- Control 2 N=6
- Pompe 1 N=6
- Pompe 2 N=3

**D** Conjugated Ubiquitin
- Total protein
- C1, C2, P1, P2

**E** Density Ratio
- p62/GAPDH
- Conj-Ub/GAPDH

Control N=4
- Pompe N=4

Downloaded from http://www.jbc.org/ by guest on July 16, 2017
Figure 7

A

<table>
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<th>2 days CQ Tx</th>
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LAMP1

B

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C

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kDa: 100, 75
Figure 8

A

Control 1 No Tx  |  Control 1 BFA Tx  |  Pompe 1 No Tx  |  Pompe 1 BFA Tx

Golgin97  |  GM130

B

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kDa

150  | 100  | 75  |  75 |

LAMP1  |  LAMP2  | GAPDH

C

Control 2 No Tx  |  Sucrose Tx

D

FB  |  iPS

C1  |  P2  |  C2  |  P1

kDa

150  | 100  |  150  |  100 |

LAMP1  |  LAMP2
Figure 10

A

B

% Intensity

% Intensity

Mass (m/z)

1579.6 1783.7

1345.6 1579.6 1783.7

1905.7 1987.8 2039.8 2109.8 2080.8 2243.8 2314.8

1905.7 1987.8 2109.8

2191.8 2395.9

2214.8

2488.9

2517.9

2604.9

2645.9

2735.0

2850.0

0 50 100

0 50 100

1200 1560 1920 2280 2640 3000
Figure 11

A

B

1783.7
2080.8
2243.8
2284.8
2314.8
2517.9
2645.9
2735.0
2850.0
2967.0
1905.7
1987.8
2109.8
2395.9
2604.9

1345.6
1579.6
1783.7
1905.7
1987.8
2109.8
2191.8
2395.9
2604.9
2967.0

% Intensity
% Intensity

Mass (m/z)

1200
1560
1920
2280
2640
3000
Golgi glycosylation defect in Pompe iPSC-cardiomyocytes

Figure 12

A

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- IIH6c4
- VIA4-1
- β-DG

B

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- Laminin Overlay
- β-DG

C

- IIH6c4/βDG
- VIA4-1/βDG
- Laminin/βDG

D

- Control 2
- No Tx
- αDG
- Laminin
- Composite

E

- Pompe 2
- No Tx
- αDG
- Laminin
- Composite
Pompe disease results in a Golgi-based glycosylation deficit in human induced pluripotent stem cell-derived cardiomyocytes
Kunil K. Raval, Ran Tao, Brent E. White, Willem J De Lange, Chad H. Koonce, Junying Yu, Priya S. Kishnani, James A. Thomson, Deane F. Mosher, John C. Ralphe and Timothy J. Kamp

J. Biol. Chem. published online December 8, 2014

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