

THE EFFECTS OF LAFORIN, MALIN, STBD1, AND PTG DEFICIENCIES
ON HEART GLYCOGEN LEVELS IN POMPE DISEASE MOUSE MODELS

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Submitted to the faculty of the University Graduate School

in partial fulfillment of the requirements

for the degree

Master of Science

in the Department of Biochemistry and Molecular Biology,

Indiana University

August 2015

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Master of Science.

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DEDICATION

For Tyler David Pray

Your love and endless support keep me afloat.

ACKNOWLEDGEMENTS

I would like to acknowledge those whose guidance and support has allowed me to complete this project and my degree. First, I would like to thank Dr. Peter Roach and Dr. Anna Depaoli-Roach; they have been not only insightful project advisers, but also incredibly supportive and kind mentors. I am incredibly grateful for their sharing of their expertise and encouragement along the way.

I would also like to thank the members of my lab for their constant help, understanding, and positivity. Chris Contreras, Rajbir Kaur, and most of all Dyann Segwich, who has been so wonderful to me. Day after day, she made the time to help and teach me and always did so with a smile. Additionally, I would like thank my classmates, with whom I have spent countless hours discussing biochemistry lessons, challenges in lab, occasionally successes in lab, and our lives in general. They will be invaluable friends for life. Denisse Hernandez, Rohit Veeramachaneni, and Matthew Martien.

Finally, for all of their support, I would like to thank my other committee member, Dr. Thomas Hurley, as well as the rest of the Department of Biochemistry and Molecular Biology. I would especially like to thank Mr. Jack Arthur, who was always there to help solve my many computer issues and/or to just have a pleasant afternoon chat.

Betsy Ann Conway

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LEVELS IN POMPE DISEASE MOUSE MODELS

Pompe disease (PD) is a rare metabolic myopathy characterized by loss of acid alpha-glucosidase (GAA), the enzyme responsible for breaking down glycogen to glucose within the lysosomes. PD cells accumulate massive quantities of glycogen within their lysosomes, and as such, PD is classified as a “lysosomal storage disease” (LSD). GAA-deficient cells also exhibit accumulation of autophagic debris. Symptoms of severe infantile PD include extreme muscle weakness, hypotonia, and hypertrophic cardiomyopathy, resulting in death before one year of age.

Certain LSDs are currently being successfully treated with enzyme replacement therapy (ERT), which involves intravenous infusion of a recombinant enzyme to counteract the endogenous deficiency. ERT has been less successful in PD, however, due to ineffective delivery of the recombinant enzyme. Alternatively, specific genes deletion may reduce lysosomal glycogen load, and could thus be targeted in PD therapy development. Absence of malin (EPM2B) or laforin (EPM2A) has been proposed to impair autophagy, which could reduce lysosomal glycogen levels. Additionally, deficiency of Stbd1 has been postulated to disable lysosomal glycogen import. Furthermore, Ptg deficiency was previously reported to abrogate Lafora body formation and correct neurological abnormalities in Lafora disease mouse models and could have similar effects on PD pathologies.

The goal of this study was to characterize the effects of homozygous disruption of *Epm2a*, *Epm2b*, *Stbd1*, and *Ptg* loci on total glycogen levels in PD mouse model heart tissue, as in severe infantile PD, it is accumulation of glycogen in the heart that results in fatal hypertrophic cardiomyopathy. *Gaa*^{-/-} mice were intercrossed with *Epm2a*^{-/-}, *Epm2b*^{-/-}, *Stbd1*^{-/-}, and *Ptg*^{-/-} mice to generate wildtype (WT), single knockout, and double knockout mice. The results indicated that *Gaa*^{-/-} hearts accumulated up to 100-fold more glycogen than the WT. These mice also displayed cardiac hypertrophy. However, deficiency of *Epm2a*, *Epm2b*, *Stbd1*, or *PTG* in the *Gaa*^{-/-} background did not reveal changes of statistical significance in either heart glycogen or cardiac hypertrophy. Nevertheless, since total glycogen was measured, these deficiencies should not be discarded in future discussions of PD therapy, as increasing sample sizes and/or distinguishing cytosolic from lysosomal glycogen content may yet reveal differences of greater significance.

Peter J. Roach, Ph.D.

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LIST OF ABBREVIATIONS

AGL	Debranching enzyme
AMP	acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
ATG	Autophagy-related protein
ATP	Adenosine triphosphate
BE	Branching enzyme
cAMP	Cyclic adenosine monophosphate
CBM20	Carbohydrate-binding module 20
DBE	Debranching enzyme
DKO	Double knockout
EPM2A	Epilepsy, progressive myoclonus, type 2A (also known as Laforin or Lafora disease)
EPM2B	Epilepsy, progressive myoclonus, type 2B (also known as Malin or NHLRC1)
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
G1P	Glucose-1 phosphate
G6P	Glucose-6 phosphate
G6Pase	Glucose-6 phosphatase
G6PDH	Glucose-6 phosphate dehydrogenase
GAA	Acid alpha-glucosidase

Glc	Glucose
GLKO	Double knockout of <i>Gaa</i> ^{-/-} and <i>Epm2a</i> ^{-/-}
GLUT	Glucose transporter
GMKO	Double knockout of <i>Gaa</i> ^{-/-} and <i>Epm2b</i> ^{-/-}
GN	Glycogenin
GPh	Glycogen phosphorylase
GPKO	Double knockout of <i>Gaa</i> ^{-/-} and <i>PTG</i> ^{-/-}
GS	Glycogen synthase
GSK3	Glycogen synthase kinase 3
GSKO	Double knockout of <i>Gaa</i> ^{-/-} and <i>Stbd1</i> ^{-/-}
HK	Hexokinase
KO	Knockout
KOH	Potassium hydroxide
LB	Lafora body
LC3	Microtubule-associated protein light chain 3
LD	Lafora disease
LiCl	Lithium chloride
LKO	Laforin knockout
LSD	Lysosomal storage disease
MgCl ₂	Magnesium chloride
MKO	Malin knockout
Na ₂ SO ₄	Sodium sulfate

NaCl	Sodium chloride
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
(NH ₄) ₂ SO ₄	Ammonium sulfate
NHLRC1	NHL repeat-containing E3 ubiquitin protein ligase 1 (also known as Malin or EPM2B)
PD	Pompe disease
PDK1	Pyruvate dehydrogenase kinase 1
PGM	Phosphoglucomutase
Ph	Phosphorylase
Ph Kinase	Phosphorylase kinase
Pi	Phosphate
PI3K	Phosphatidylinositol kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKB	Protein kinase B (also known as AKT)
PKO	PTG knockout
PLP	Pyridoxal phosphate
PP1	Protein phosphatase 1
PTG	Protein targeted to glycogen

R _{GL}	Regulatory targeting subunit
rhGAA	Recombinant human acid alpha-glucosidase
RT	Room temperature
SEM	Standard error of the mean
SKO	Stbd1 knockout
SRT	Substrate replacement therapy
Stbd1	Starch-binding domain-containing protein 1
UDP	Uridine diphosphate glucose
SKO	Stbd1 knockout
WT	Wildtype

INTRODUCTION

1. Glycogen Structure

Glycogen is the large, branched polymer of glucose that is the primary form of energy storage in animals and fungi. It is analogous to starch, the glucose storage unit in plants. In the body's fed state, insulin signals to cells to store excess glucose from the meal as glycogen. The two primary sites of glycogen metabolism are the liver and skeletal muscle, though other tissues, including heart, adipose, brain, and kidney, are also capable of synthesizing glycogen (1). Glycogen polymerization occurs between glucose monomers via α -1,4-glycosidic linkages, while branch points are introduced via α -1,6-glycosidic linkages (Figure 1). As a polymer, glycogen has unbranched outer A-chains and branched inner B-chains, each of which has approximately two branch points (Figure 2). In totality, one molecule of glycogen consists of around 55,000 glucose residues, making for a molecular mass of $\sim 10^7$ kDa and a diameter of ~ 44 (nm) (2,3). Glycogen is primarily composed of glucose, but also contains small amounts of glucosamine and phosphate (4-6). Glycogen phosphorylation has been more intently studied and has been accepted as an integral part of glycogen architecture. Frequency of glycogen phosphorylation has been measured to be one phosphate for every ~ 650 glucose residues in rabbit skeletal muscle glycogen and one for every ~ 2000 residues in mouse muscle glycogen (7,8). The functions of glycogen phosphate and glucosamine have yet to be fully characterized; glycogen hyper-phosphorylation, however, has been shown to be associated with the neurodegenerative disorder, Lafora disease (see Introduction, section 4). As glycogen molecules grow, they may accumulate chemical

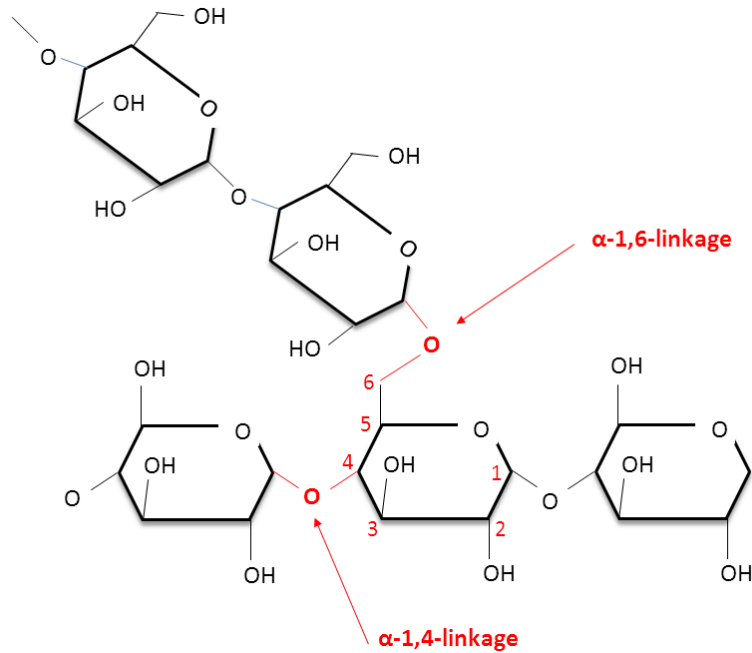


Figure 1. Glycosidic Linkages in Glycogen. Glycogen synthase catalyzes glycogen polymerization via α -1,4-glycosidic linkages. Glycogen branching enzymes introduce branches every ~ 12 glucose units via α -1,6-glycosidic linkages. The numbering of the carbons is shown for one of the glucose units. Adapted from “Lafora Disease: Mechanisms Involved in Pathogenesis” by Punitee Garyali, 2014.

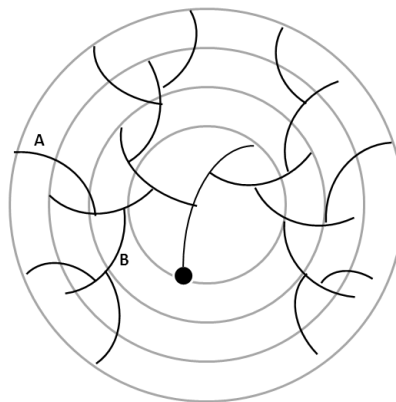


Figure 2. Glycogen Structure. Glycogen organization depicted in a tiered model: the outer A-chains are unbranched, while the branched inner B-chains have an average of two branches. Glycogenin is represented as a black dot. Adapted from “Glycogen and its metabolism: some new developments and old themes” by Roach et al, 2012.

and structural abnormalities, which result in abnormal metabolism or solubility.

Deposits of insoluble glycogen-like material have been observed in various neurological diseases.

There are certain glycogen-associated proteins, which bind to glycogen, each other, and/or intracellular membranes; the complexes of these proteins along with glycogen are termed 'glycogen particles' (9-15) (Figure 3). Known glycogen-associated proteins include the glycogen synthesis initiator enzyme, glycogenin, and glycogen metabolism-mediating enzymes, glycogen synthase, glycogen phosphorylase, glycogen-branching enzyme, debranching enzyme, as well as various regulatory enzymes, such as phosphorylase kinase and the protein phosphatase 1G (PP1G) family (16). The β -subunit of AMP-activated protein kinase (AMPK), laforin, and starch-binding domain containing protein 1 (Stbd1) have also been shown to interact with glycogen via carbohydrate-binding module 20 domains (CBM20) (17-24). Functional roles in glycogen metabolism have been proposed for each of these glycogen-associated proteins.

2. Glycogen Metabolism

The polymerization of glycogen, called glycogenesis, requires glucose from either carbohydrate intake or gluconeogenic precursors (25). Triggered by glucose uptake via glucose transporters (GLUTs) (26), glycogenesis (Figure 4) requires that glucose first be converted to glucose-6-phosphate (G6P). Then, phosphoglucomutase (PGM) converts G6P to glucose-1-phosphate (G1P), which is in turn converted to uridine diphosphate glucose (UDPG) by UDP glucose phosphorylase (UGP). The self-glycosylating protein,

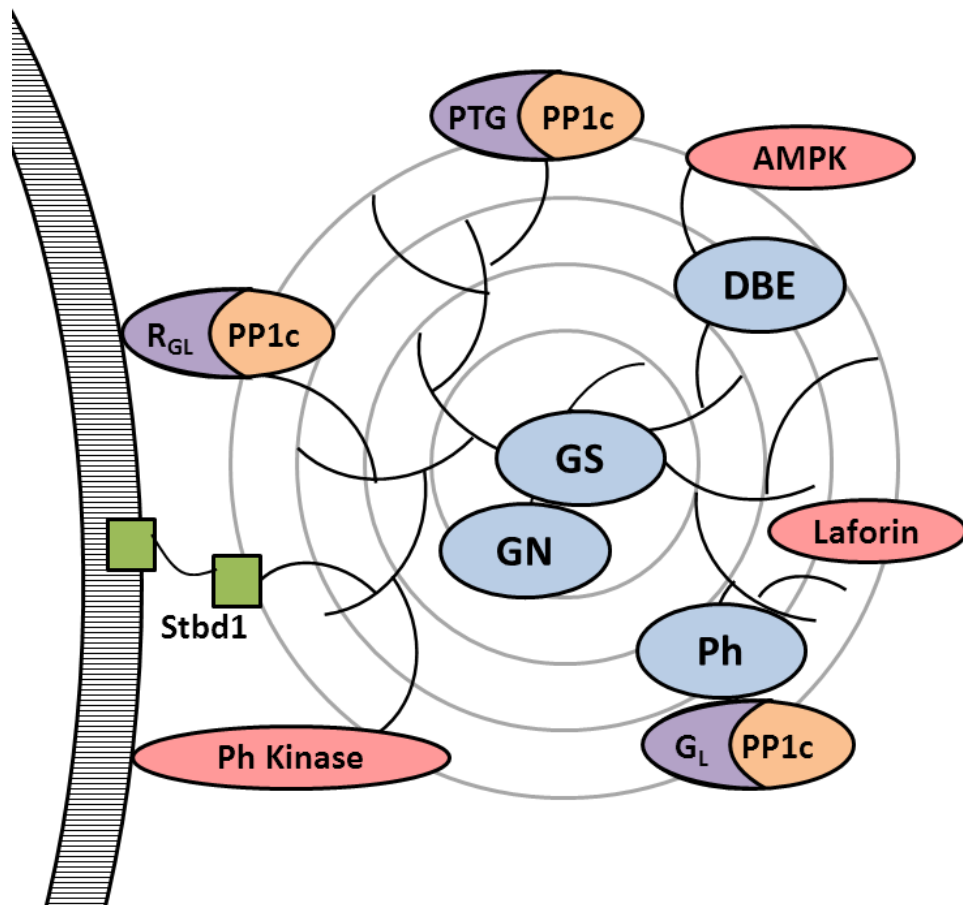


Figure 3. Glycogen Particles. The glycogen-associated proteins: the metabolic enzymes glycogenin (GN), glycogen synthase (GS), phosphorylase (Ph), and debranching enzyme (DBE); phosphatase type 1 catalytic subunit (PP1c); glycogen-targeting subunits R_{GL}, G_L, and PTG; the protein kinases AMPK and phosphorylase kinase (Ph Kinase) ; Laforin; and Stbd1. Capable of membrane anchoring are Stbd1, Ph Kinase, and R_{GL}. Adapted from “Glycogen and its metabolism: some new developments and old themes” by Roach et al, 2012.

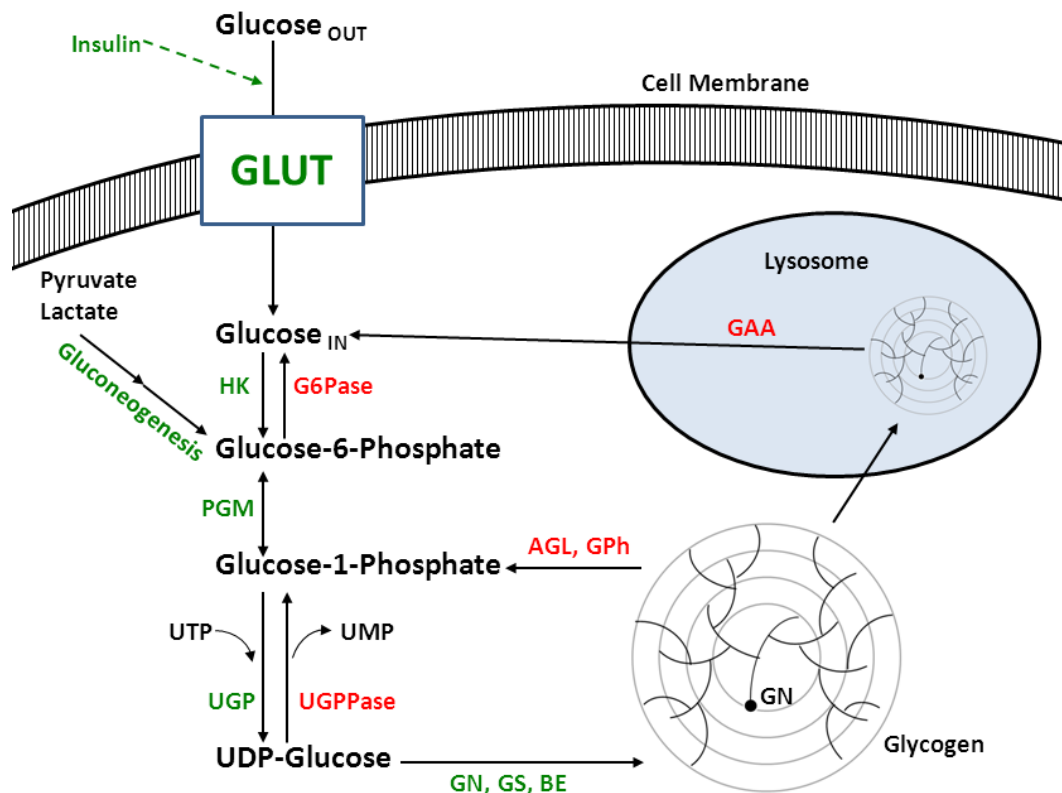


Figure 4. Overview of Glycogen Metabolism. GLUT, glucose transporter; HK hexokinase; G6Pase, glucose-6-phosphatase; PGM, phosphoglucomutase; UGP, UDP-glucose-phosphorylase; UGPPase, UDP-glucose-pyrophosphatase; GN, glycogenin; GS, glycogen synthase; BE, branching enzyme; AGL, debranching enzyme; GPh, glycogen phosphorylase; GAA, acid- α -glucosidase. Adapted from “Lafora Disease: Mechanisms Involved in Pathogenesis” by Punitee Garyali, 2014.

glycogenin (GN), begins glycogen polymerization by forming a short polymer of glucose units from UDPG (27-29). Glycogen synthase (GS) then continues to link glucose from UDPG via α -1,4 glycosidic bonds to the non-reducing end of the growing glycogen molecule. This reaction is the rate-limiting step in glycogen synthesis. Glycogen branching enzyme (GBE) introduces branch points via an α -1,6 glycosidic bond at the C6 hydroxyl of glucose residue in the chain, giving an overall average branching frequency of one every twelve glucoses.

Conversely, glycogen may also be broken back down to reform glucose via the process of glycogenolysis (Figure 4). The cytosolic pathway involves three steps (Figure 5). First, in the rate-limiting step, a phosphoryl group substitution by glycogen phosphorylase (GPh) and its cofactor pyridoxal phosphate (PLP) breaks the position one bonds until just four glucose residues remain on the branch. Second, the debranching enzyme (AGL) catalyzes the transfer and α -1,4-glycosidic linkage of these glucose units to the end of another branch of the glycogen polymer. Third and finally, the C-terminus of AGL, which possesses amylo-1,6-glucosidase activity, removes the last remaining glucose residue.

Hormonal regulation of glycogen metabolism is mediated by covalent modification of and allosteric ligand-binding to the primary glycogenic and glycogenolytic enzymes (Figure 6). Following a meal, the increase in blood glucose signals to the pancreatic β -cells to release insulin into the blood stream. Insulin binds allosterically to either of the two insulin receptor α -subunits, which causes a conformational change of the receptor and trans-phosphorylation of the two β -

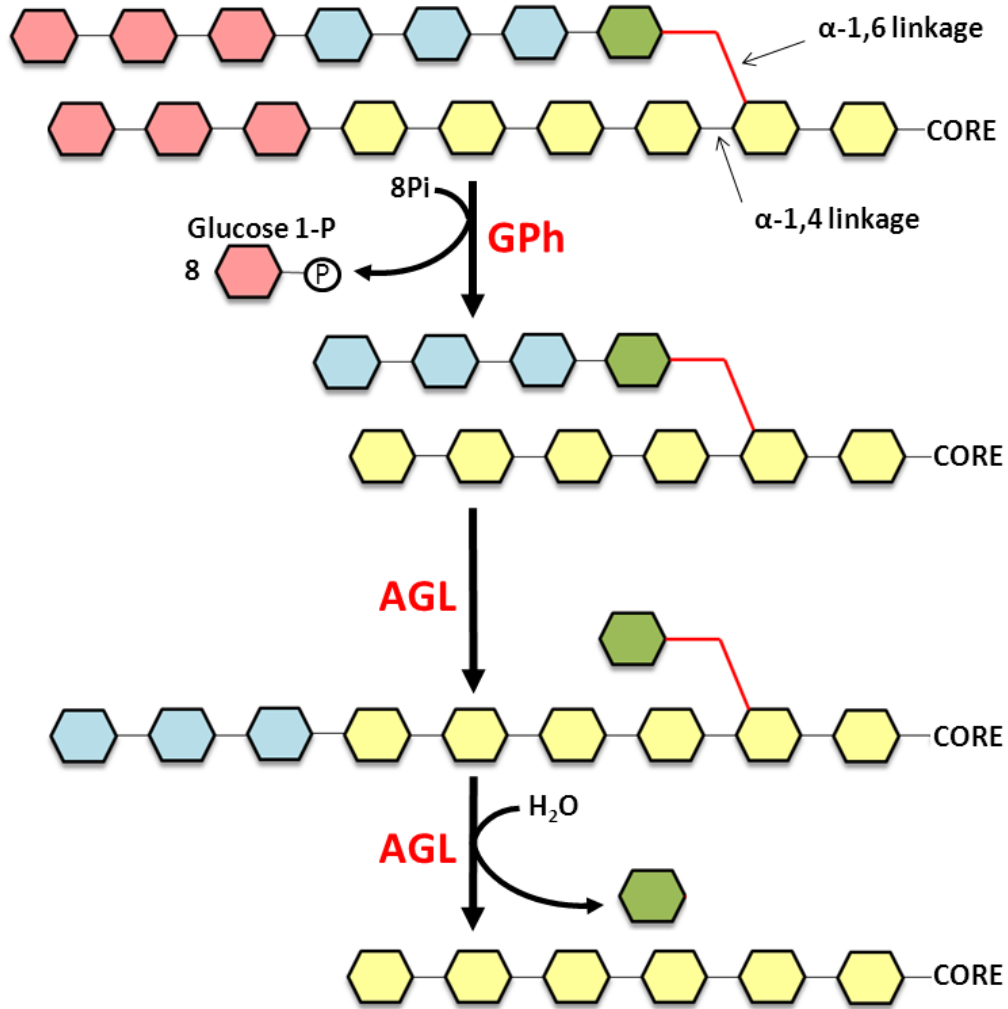


Figure 5. Cytosolic Glycogenolysis. Glycogen phosphorylase (GPh) breaks glycosidic linkages until just four glucose residues remain on the branch; debranching enzyme (AGL) transfers and these glucose units to the end of another branch; AGL, removes the last glucose residue. Adapted from Berg JM, Tymoczko JL, Stryer L. Biochemistry. 5th edition. New York: W H Freeman; 2002.

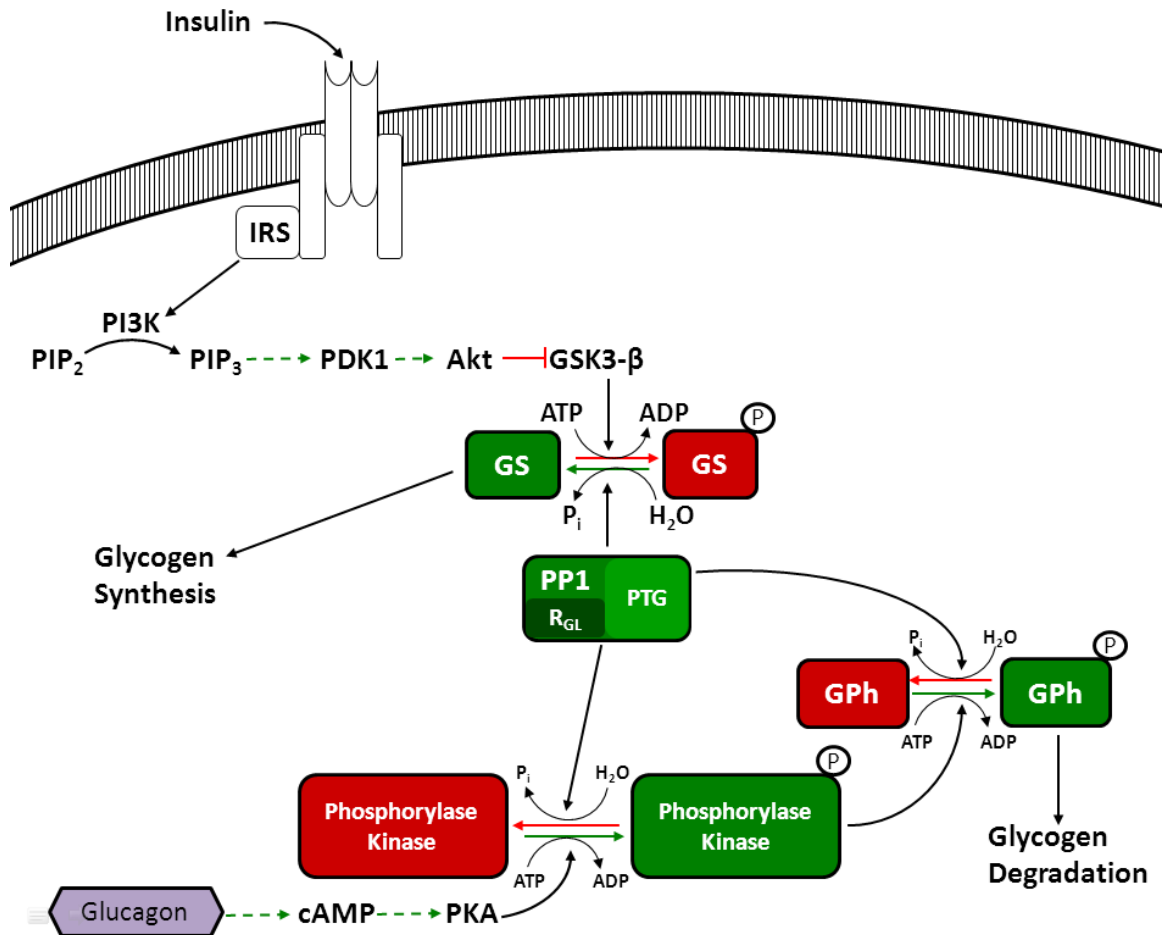


Figure 6. Regulation of Glycogen Metabolism. Schematic representation of the primary signaling pathways involved in glycogen metabolism. PI3K, phosphatidylinositol kinase; PIP₂, phosphatidylinositol-4,5-bisphosphate; PIP₃, phosphatidylinositol-3,4,5-triphosphate; GSK3, glycogen synthase kinase 3; GS, glycogen synthase; PP1, protein phosphatase 1; PTG, protein targeting to glycogen; R_{GL}, regulatory targeting subunit; GPh, glycogen phosphorylase; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate. Adapted from “Lafora Disease: Mechanisms Involved in Pathogenesis” by Punitee Garyali, 2014.

subunits. The activated, phosphorylated cytosolic tyrosine kinase phosphorylates substrates that recruit SH2 domain-containing proteins, such as phosphatidylinositol-3-kinase (PI3K). Upon activation, PI3K converts membrane lipid, phosphatidylinositol-4,5-bisphosphate (PIP₂), to phosphatidylinositol-3,4,5-triphosphate (PIP₃), which in turn activates protein kinase B (PKB, also known as AKT) via activation of PDK1. Activated AKT inhibits glycogen synthase kinase-3 (GSK3) via phosphorylation, thereby preventing GSK3-mediated glycogen synthase inactivation (30). Conversely, if activated, GSK3 phosphorylates and inhibits glycogen synthase (1). GS is activated by protein phosphatase type I (PP1) via dephosphorylation, which functions in conjunction with glycogen-targeting subunits, such as regulatory-targeting subunit (R_{GL} or G_M) and protein targeting to glycogen (PTG). PP1 also regulates glycogen phosphorylase (GPh) via phosphorylation-mediated inactivation, thereby decreasing glycogen degradation. When glycogen is being degraded, GPh is activated by phosphorylase kinase, which is in turn activated by PKA and deactivated by PP1 and PP2A. In short summary, pro-glycogenic signaling via insulin facilitates glycogen synthesis by inhibiting GSK3, the inhibitor of GS, while pro-glycogenolytic signaling via epinephrine or glucagon facilitates glycogen breakdown by increasing cAMP-mediated PKA activity.

3. Lysosomal Disposal of Glycogen and Pompe Disease

While the primary means of glycogen degradation occurs via the cytosolic pathway, some glycogen is also transported into the lysosomes to be cleaved hydrolytically by the lysosomal enzyme acid α -glucosidase (GAA) (Figure 4). Although

the lysosomal pathway is not the foremost mode of glycogen degradation, deficiency of GAA leads to a severe lysosomal storage disease known as Pompe disease (OMIM 232300) (31). The exact mode by which glycogen is transported into the lysosomes is unknown; it is thought, however, to involve autophagy-like vesicle import. Under stress or nutritional deprivation, cells use autophagy as a catabolic mechanism for recycling cellular materials that can be redistributed (32,33). Autophagy, specifically macroautophagy, involves engulfment of isolated cytoplasmic constituents within double-membraned vesicles, known as autophagosomes (Figure 7). Autophagosomes may fuse with lysosomes, thereby forming autophagolysosomes, and ultimately the cargo is degraded by lysosomal enzymes (34). Employed in these processes of autophagosome formation, delivery, and lysosomal fusion are several crucial autophagy-related proteins (ATGs) (35).

Studies have indicated a potentially significant link between lysosomal glycogen degradation and autophagy. In one such study, 'glycogen autophagy' is described in the livers of newborn infants (36). Prenatal glycogen accumulation is utilized via lysosomal degradation as the primary energy source immediately postnatal before gluconeogenic processes have fully developed. In another study, Pompe disease (PD) mouse models (*Gaa*^{-/-}) show massive accumulation of lysosomal glycogen, as well as a significant increase in the number and size of glycogen-containing autophagosomes, an occurrence known as 'autophagic build-up' (37). Raben et al (38) have created *Gaa*^{-/-} mouse models with skeletal muscle-specific deficiency of autophagy-related protein 7 (Atg7);

results showed reduced skeletal muscle autophagic build-up in the animals, but worsened overall clinical phenotype.

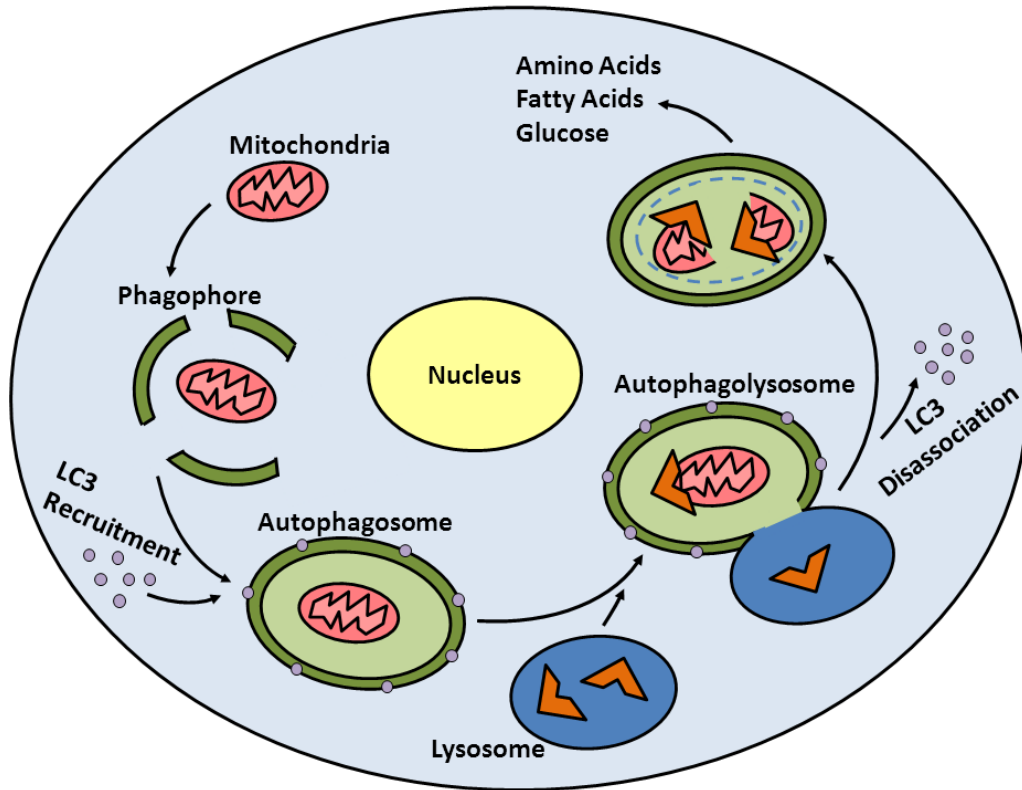


Figure 7. Autophagy. Schematic representation of macroautophagy. Specific cytoplasmic constituents, for example a mitochondrion, are engulfed by a lipid double membrane, into which microtubule associated protein 1 light chain 3 (LC3-1) is incorporated, thereby forming the autophagosome. The autophagosome fuses to and transfers contents into a lysosome, thus forming an autophagolysosome, wherein cargo is degraded by lysosomal enzymes. Adapted from “Lafora Disease: Mechanisms Involved in Pathogenesis” by Punitee Garyali, 2014.

Pompe disease (PD), also called glycogen storage disease type II (GSDII), is a rare metabolic myopathy caused by loss of acid α -glucosidase (GAA), the lysosomal enzyme responsible for breaking glycogen down into glucose within lysosomes via glycosidic bond cleavage (39). The hallmark pathology of this disease is massive accumulation of lysosomal glycogen, and as such, PD is classified as a 'lysosomal storage disease' (LSD) (39,40). Glycogen accumulation has also been detected in autophagosomes and late endosomes, which is referred to as 'autophagic buildup.' In PD patients, lysosomal glycogen accumulation occurs in several tissue types, though cardiac and skeletal tissues predominantly contribute to clinical manifestation. Primary symptoms include muscle weakness, hypotonia, cardiac hypertrophy, and cardiomyopathy. In milder, later-onset adult PD cases, GAA enzyme activity is reduced by about 80-90%, and death is often attributed to respiratory failure. In the more severe, infantile PD cases, GAA enzyme activity is reduced by 98-99%, and death due to hypertrophic cardiomyopathy usually occurs within the first year of life (31,41). Analyses of *Gaa*^{-/-} mouse models reveal heart sizes up to three times larger than normal (42), which is due in large part to markedly increased left ventricular wall thickness (43). As demonstrated by Douillard-Guilloux et al (44), PD-related heart failure is likely directly related to the massive glycogen accumulation within the cells of heart tissue. In this study, *Gaa*^{-/-} mice were intercrossed with glycogen synthase 1 knockout mice (*Gys1*^{-/-}) to generate a new double-knockout model (DKO). The DKO animals showed significantly reduced levels of glycogen in the heart and skeletal muscle, and reduced lysosomal distension and autophagic build-up, as well as complete reversal of cardiomegaly. Furthermore, this

Gaa^{-/-} *Gys1*^{-/-} mouse model also exhibited improved glucose metabolism and insulin tolerance.

The only treatment currently available for PD is enzyme replacement therapy (ERT), which involves intravenous infusion of a recombinant human GAA (rhGAA; alglucosidase alfa, Myozyme[®], Genzyme Corporation, Framingham, MA) to counteract the endogenous deficiency. ERT has been less successful in PD, however, due to inefficient delivery of the recombinant enzyme to the lysosomes of certain target tissues, in particular skeletal muscle (45). Furthermore, these treatments are expensive and must be administered continuously throughout the life of the patient. As such, there is a significant need for improvement in Pompe disease therapy.

4. Lafora Disease

First characterized in 1911 by Spanish neurologist Gonzalo Lafora, Lafora progressive myoclonus epilepsy, or Lafora disease (LD), is a fatal neurodegenerative disease characterized by deposits of aberrantly branched glycogen, called polyglucosan, and some associated proteins (46) (Figure 8). These dense inclusions were later named 'Lafora bodies' (47) and have been detected microscopically in several tissue types, including skeletal muscle, neuron, and heart (46,48). In adolescence, LD manifests as myoclonus, epilepsy, and neurodegeneration, and as the disease progresses, the patient generally enters a vegetative state and dies due to respiratory failure within a decade (49,50). This disease is very rare and occurs more often in areas of the world where consanguinity is common.

Lafora disease is primarily caused by mutation of either the gene encoding laforin, *EPM2A* (23,51), or malin, *EPM2B* (52). The resultant disease phenotype of *EPM2A* mutation is indistinguishable from that of *EPM2B* mutation (53); however, patients with *EPM2B* mutations experience later onset of symptoms and slower disease progression (54). To link Lafora body formation with Lafora disease pathology, *Epm2a*^{-/-} mice were crossed with *Ptg*^{-/-} mice (55), thereby generating an LD mouse model deficient in PTG, a critical activator of glycogen synthesis via regulation of glycogen synthase (see section 8). This resulted in near complete alleviation of molecular LD pathogenesis; the double knockout mice had almost no Lafora bodies, no neurodegeneration, and no seizures. Echocardiographic analyses of both *Epm2a*^{-/-} and *Epm2b*^{-/-} mice indicate no significant impairment of cardiac function (56).

5. Laforin

Encoded by *EPM2A*, laforin is a ubiquitous, glycogen phosphatase most highly expressed in the skeletal muscle, liver, kidneys, heart, and brain (57,58). Laforin contains both an N-terminal carbohydrate-binding (CBM20) module and a catalytic C-terminal atypical dual-specificity phosphatase domain within the same polypeptide. In accordance with this architecture, recombinant laforin binds to complex polysaccharides (59,60), and in addition, laforin has been shown *in vitro* to remove the phosphate that was incorporated during synthesis from both glycogen (9) and amylopectin (62).

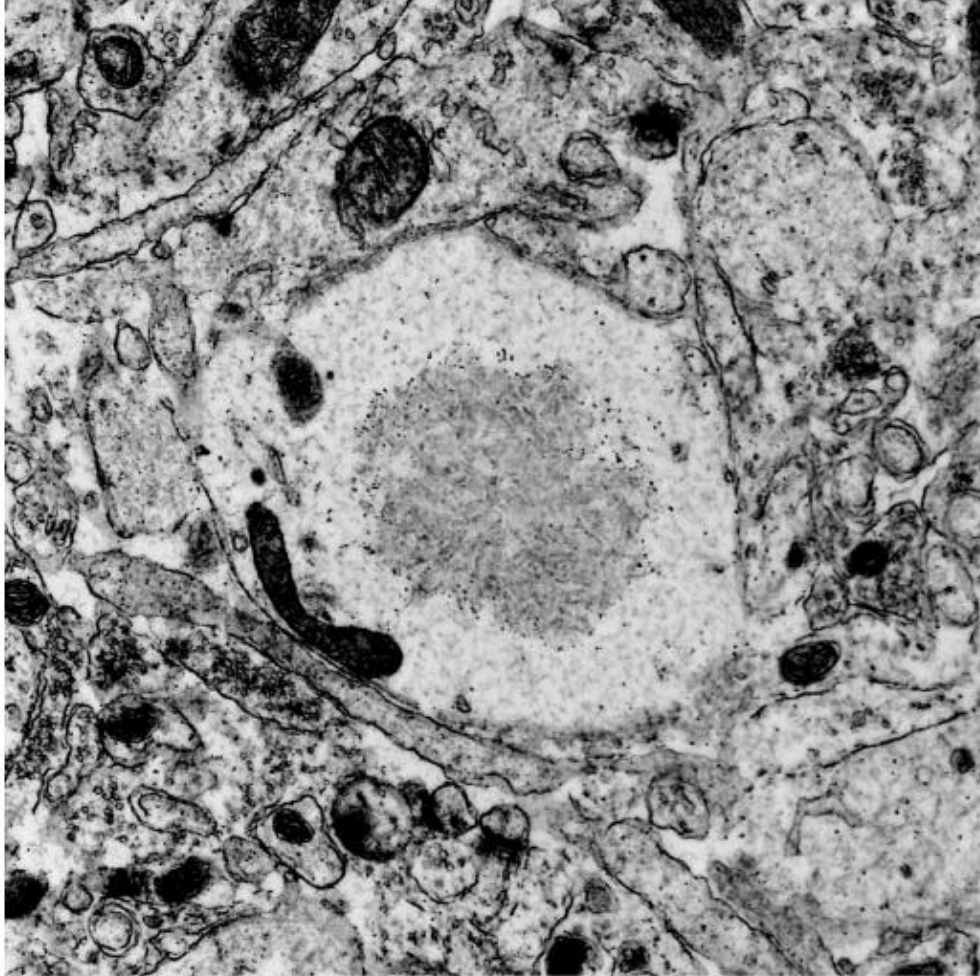


Figure 8. Lafora Bodies. An electron micrograph depiction of a Lafora body in a neuropil of an *Epm2a*^{-/-} mouse. Courtesy of Cameron Ackerley, The Hospital for Sick Children, University of Toronto.

While trace amounts of phosphate are found in normal glycogen, both *Epm2a*^{-/-} (7,8) and *Epm2b*^{-/-} (61,62) (see section 6) mice exhibit hyperphosphorylated glycogen, which results in structural abnormalities consistent with Lafora body formation as the mice age. These results indicate that laforin functions as a glycogen phosphatase *in vivo*. Around 60 disease-causing mutations have been identified within *EPM2A*, and nearly all result in a defect of either the phosphatase or carbohydrate binding activities of laforin (The Lafora Progressive Myoclonus Epilepsy Mutation and Polymorphism Database; <http://projects.tcag.ca/lafora/>). Malin and laforin have been implicated in glycogen metabolism as well as quality control via protein degradation, the autophago-lysosomal pathway, the ubiquitin-proteasomal pathway, and the ER stress response pathway (63-65). Furthermore, laforin has also been shown to bind to several of the enzymes involved in glycogen metabolism: glycogen synthase, GSK3, PTG, and malin (66-68). It has been proposed that laforin senses cytosolic glycogen accumulation, and subsequently alters its own cellular levels, as necessary (69). Although the specific details of these functions and interactions are unclear, it is now widely accepted that laforin acts as a glycogen phosphatase.

6. Malin

EPM2B (also known as *NHLRC1*) is located on chromosome 6q22.3 and contains only one exon, which encodes the 395 amino acid polypeptide known as malin. It consists of a RING finger characteristic of E3 ubiquitin ligases and 6 NHL domains involved in protein-protein interactions (52). Interspersed throughout the *EPM2B* gene, in both the

RING finger and NHL domains, are nearly 60 disease-causing mutations and polymorphisms (The Lafora Progressive Myoclonus Epilepsy Mutation and Polymorphism Database; <http://projects.tcag.ca/lafora/>).

From *in vitro* and overexpressing cell culture systems, EPM2B has been proposed to ubiquitinate enzymes involved with glycogen metabolism, including laforin (68), glycogen synthase (GS) (70), protein targeting glycogen (PTG) (68,70), and glycogen debranching enzyme (AGL) (71). If ubiquitination by malin served to target any of these proteins for degradation, it could be expected that malin deficiency would increase these proteins levels *in vivo*. In *Epm2b*^{-/-} mice, however, it was found that the levels of each these proteins remained unchanged, except laforin, which was increased (72). Increased levels of laforin have also been detected in human Lafora disease patients with *EMP2B* mutations (73). While malin deficiency may be preventing degradation of laforin, it has also been proposed that laforin is simply binding to and being sequestered by abnormal glycogen, thus prohibiting its degradation (74). In fact, in malin-deficient mice, genetic depletion of PTG (see section 8) reduced glycogen production and almost completely eliminated Lafora bodies (75). In addition to resolving the neurodegeneration, myoclonus, seizure susceptibility, and behavioral abnormality, laforin levels were also normalized (DePaoli-Roach et al, unpublished results) arguing against the idea that laforin is a malin substrate. Similar results were also reported in studies of specific deletion of glycogen synthase in the brain tissue of mice (76). While much remains to be understood regarding malin's exact function, downregulation of

glycogen synthesis seems to be a potential therapy for both malin and laforin deficiency-linked Lafora disease.

7. Stbd1

Starch binding domain containing protein 1 (Stbd1, also known as GENX 3414 and genethonin 1) is highly expressed in the primary glycogen-storing tissues, heart, liver, and skeletal muscle (21,77,78). The N-terminus of Stbd1 enables membrane association via a highly conserved 24-residue hydrophobic sequence, while the C-terminus contains a carbohydrate-binding CBM20 domain (18,21,77,78). Stbd1 binds preferentially to less-branched polysaccharides, such as the glycogen isolated from Lafora disease mouse models (21).

Although the exact function of Stbd1 is unknown, recent studies suggest that Stbd1 plays a role in glycogen metabolism, functioning as tether for glycogen to subcellular membranes (21). Additionally, studies using glycogen-deficient mice (glycogen synthase knockouts, *Gys1*^{-/-}) indicate that Stbd1 levels are significantly decreased. Together these results suggest an important link between Stbd1 and the intracellular trafficking and disposal of aberrantly-structured glycogen. In another study using Pompe disease mice with abnormal glycogen accumulation, Stbd1 levels were quantified and shown to be elevated (79). Suppression of Stbd1, however, did not significantly attenuate lysosomal glycogen accumulation, and as such, the authors conclude that Stbd1 is not a likely therapeutic target for PD.

8. PTG

Encoded by the *PPP1R3C* gene, protein targeting glycogen (PTG) is expressed primarily in insulin-sensitive tissues and serves as a scaffolding protein that facilitates localization of protein phosphatase-1 (PP1) to glycogen (80). PP1 plays a central role in glycogen synthesis and is composed of a catalytic subunit responsible for dephosphorylation of proteins and a regulatory subunit responsible for compartmentalization and determination of substrate specificity. This enzyme enhances glycogen synthesis via glycogen synthase activation, while inhibiting glycogen breakdown via deactivation of phosphorylase kinase and glycogen phosphorylase.

In a previous study, disruption of *PPP1R3C* was reported to be embryonic lethal (81). In contrast, however, is more recent work by DePaoli-Roach et al, in which a PTG-deficient mouse line was generated and found to be healthy with no obvious defects. These observations are supported by the studies demonstrating that crosses of *Ptg*^{-/-} mice with either *Epm2a*^{-/-} or *Epm2b*^{-/-} mice result in healthy, viable double knockout animals (62,75).

9. Mouse Model of Pompe Disease

Genotypically and phenotypically accurate PD mouse models deficient in lysosomal acid α -glucosidase were generated as previously described by Raben et. al (82). The *Gaa*^{-/-} mice possess characteristics of both infantile and adult PD. The animals develop cardiomegaly, cardiomyopathy, and skeletal muscle myopathy, while other symptoms, such as muscle wasting, manifest relatively late at around 7 – 9

months of age (83). *Gaa*^{-/-} mice survive up to 1.5 years and accurately recapitulate the biochemistry of the disease. As such, this mouse model is suitable for further investigation of the pathophysiology of and novel therapies for Pompe disease.

RESEARCH OBJECTIVE

The primary aim of this study is to determine the molecular effect of four specific genetic knockouts, *Epm2a*, *Epm2b*, *Stbd1*, and *Ptg*, on total glycogen load in the heart tissue of Pompe disease mouse models. We opted for examination of the heart, as in severe infantile PD, it is accumulation of glycogen in this tissue that results in mortality due to hypertrophic cardiomyopathy. Malin and laforin were selected for this study with the hypothesis that their absence might reduce autophagy, which consequently could also decrease lysosomal glycogen importation and accumulation. *Stbd1* was chosen with the rationale that its deficiency might disable glycogen transport into lysosomes, thereby alleviating the major pathology of Pompe disease. Lastly, PTG was selected in light of the previously described success of PTG deficiency in abrogating Lafora body formation in LD mouse models (55,75). Glycogen levels were examined in the hearts of wildtype (WT), single knockout, and double knockout mice in a *Gaa*^{-/-} background.

EXPERIMENTAL PROCEDURES

1. Maintenance of Mice

The Pompe disease mouse model (*Gaa*^{-/-}) used to generate the mouse lines in this study was obtained from the laboratories of Dr. Nina Raben at the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) (82). The malin and laforin deficient (*Epm2a*^{-/-} and *Epm2b*^{-/-}) mice have been described previously (56,73,84). The *Stbd1* deficient mice (*Stbd1*^{-/-}) were recently generated (unpublished results) in the laboratory of Dr. Peter Roach, Department of Biochemistry and Molecular Biology at Indiana University School of Medicine. The PTG deficient mice (*Ptg*^{-/-}) were generated in the laboratory of Dr. Anna Depaoli-Roach in the Department of Biochemistry and Molecular Biology at Indiana University School of Medicine (74). The *Gaa*^{-/-} mice were intercrossed with the *Epm2a*^{-/-}, *Epm2b*^{-/-}, *Stbd1*^{-/-}, and *PTG*^{-/-} mice to generate wildtype, single knockout, and double knockout mice.

All mice used in this study were maintained in temperature and humidity-controlled environments with a 12:12-hour light-dark cycle, fed a standard murine diet (Harlan Teklad global diet 2018SX), and allowed food and water *ad libitum*. After weighing, 10-12 month-old male animals were sacrificed by cervical dislocation. Heart tissue was harvested, transferred immediately into liquid nitrogen, stored at -80°C, and weighed at a later time. All studies were conducted in strict accordance with Federal Guidelines, and all protocols were approved by the Institutional Animal Use and Care Committee of Indiana University School of Medicine.

2. Measurement of Glycogen Levels in Tissue Samples

Total glycogen in the heart tissue samples was quantified following the protocol developed by Suzuki et al (85). Approximately 30mg of powdered heart tissue was measured into a 1.5 mL screw cap tube and 300 μ L of boiling hot, freshly prepared 30% KOH was added. The tissue was boiled for 30 minutes, mixing by inversion every 5-10 minutes. The samples were cooled on ice, and then 100 μ L of 2% Na₂SO₄ (w/v), 10 μ L of 1M LiCl, and 800 μ L of 100% ethanol were added. The samples were incubated at -20°C overnight to precipitate the glycogen.

After precipitation, the glycogen was collected by centrifugation at 14,000rpm at 4°C for 20 minutes, dried for 10 minutes in a SpeedVac, resuspended in 100 μ L of water, precipitated again by addition of 10 μ L of 1M LiCl and 800 μ L of 100% ethanol, and incubated at -20°C for 1 hour. This procedure was repeated one more time, and the samples were kept at -20°C overnight.

Following the third precipitation, the glycogen pellet was digested enzymatically overnight in a 40°C water bath with 100 μ L of amyloglucosidase diluted in 0.2 mM sodium acetate pH 4.8 to give a final concentration 0.3mg/ml. Following the digestion, the samples were centrifuged at 14,000rpm for 5 minutes, and the supernatant was transferred to a new tube. The amount of glucose was determined by a coupled reaction: conversion of glucose to glucose-6-phosphate by hexokinase and reduction of nicotinamide adenine dinucleotide phosphate (NADP⁺) to nicotinamide adenine dinucleotide phosphate (NADPH) by glucose-6-phosphate dehydrogenase (G6PDH) (86). Unless a dilution was needed, 10 μ L of digested glycogen was added to 300 μ L of assay

buffer (300mM triethanolamine pH 7.6, 4mM MgCl₂, 0.9mM NADP⁺, 2 mM ATP, and 2μg/mL glucose-6-phosphate dehydrogenase (Roche)). From this mixture, 100μL was taken from each tube, and using a spectrophotometer, the absorbance was read at 340nm and recorded to quantify the inference of background absorption in the samples. Then, 5μL of hexokinase (1 mg/mL stock, Roche) (diluted 1:10 in 3.2M (NH₄)₂SO₄) was added to the remaining 200μL, and the samples were incubated at room temperature for 30 minutes. The absorbance was read and recorded at 340nm again to determine the concentration of NADPH, which is directly related to the glucose produced by the digestion of glycogen. The difference between the two spectrophotometric values, the net absorption, was used to calculate glucose equivalents based on a molar extinction coefficient for NADPH of 6.22 x10³. Glycogen content is expressed as μmol of glucose/mg of tissue.

3. Statistical Analysis

The data are presented as the mean ± SEM for the indicated number of animals. Statistical significance was determined by an unpaired Student's t-test and was considered significant at p < 0.05.

RESULTS

Accumulation of lysosomal glycogen, the hallmark of Pompe disease, occurs in the *Gaa*^{-/-} mouse models of the disease. To determine the effects laforin, malin, *Stbd1*, and *Ptg* deficiencies on glycogen levels in PD mouse models, we analyzed the heart glycogen content of wildtype (WT), single knockouts, and double knockouts. Hearts samples were harvested from the animals and glycogen was determined as described in the experimental procedures. Five different mice were assayed for every genetic combination.

In the first experiment, depicted in Figure 9, we quantified glycogen levels in the heart of *Epm2a*^{-/-} and *Gaa*^{-/-} mouse crosses. The glycogen levels of the *Gaa*^{+/+} *Epm2a*^{+/+} (wildtype or WT), *Gaa*^{+/+} *Epm2a*^{-/-} (laforin single knockout or LKO), *Gaa*^{-/-} *Epm2a*^{+/+} (Gaa single knockout or GKO), and *Gaa*^{-/-} *Epm2a*^{-/-} (Gaa and laforin double knockout or GLKO) groups were 1.93±0.58, 11.24±3.60, 197±3.74, and 190±7.98µmol glucose/gram tissue, respectively. The LKO group had 5-fold higher glycogen levels than the WT, and this difference reached statistical significance ($p = 0.034$). The GKO had approximately 100-fold higher glycogen content than the WT ($p = 2.2E-11$), thus also reaching statistical significance. Likewise, the GLKO group had around 90-fold higher glycogen levels than the WT, a difference which was statistically significant ($p = 1.2E-8$). The GLKO group had approximately 4% less glycogen than the GKO, however this difference was not significant ($p = 0.41$).

Figure 10 depicts the results of the second experiment, wherein we quantified glycogen levels in hearts of *Epm2b*^{-/-} and *Gaa*^{-/-} mouse crosses. The glycogen levels of

the *Gaa*^{+/+} *Epm2b*^{+/+} (wildtype or WT), *Gaa*^{+/+} *Epm2b*^{-/-} (malin single knockout or MKO), *Gaa*^{-/-} *Epm2b*^{+/+} (Gaa single knockout or GKO), and *Gaa*^{-/-} *Epm2b*^{-/-} (Gaa and malin double knockout or GMKO) groups were 3.43±0.51, 7.44±1.35, 259±15.2, 266±20.5 μmol glucose/gram tissue, respectively. The MKO had over 2-fold more glycogen than the WT, with the difference in values being statistically significant (p = 0.028). With a difference also reaching statistical significance, the GKO group had around 75-fold higher glycogen content than the WT (p = 1.6E-7). The GMKO group glycogen levels as compared to the WT were approximately 80-fold greater, which was also statistically significant (p = 1.3E-6). The difference in glycogen levels between the GKO and GMKO groups was not of statistical significance (p = 0.76).

In the third experiment, we quantified glycogen levels in heart samples from the *Stbd1*^{-/-} and *Gaa*^{-/-} mouse crosses; these results are shown in Figure 11. The glycogen levels of the *Gaa*^{+/+} *Stbd1*^{+/+} (wildtype or WT), *Gaa*^{+/+} *Stbd1*^{-/-} (*Stbd1* single knockout or SKO), *Gaa*^{-/-} *Stbd1*^{+/+} (Gaa single knockout or GKO), and *Gaa*^{-/-} *Stbd1*^{-/-} (Gaa and *Stbd1* double knockout or GSKO) groups were 2.86±0.60, 3.79±0.60, 205.3±20.91, and 196.2±16.15 μmol glucose/gram tissue, respectively. The SKO group had approximately 32% more glycogen than the WT, though the difference in values was not significant (p = 0.31). The glycogen content of both the GKO and the GSKO groups was about 70-fold higher than the WT, and both differences were statistically significant (p = 1.1E-5 and p = 2.2E-6, respectively). The GSKO group had slightly lower glycogen levels than the GKO, though the difference was not statistically significant (p = 0.74).

Figure 12 depicts the results of the final experiment, in which we quantified heart tissue glycogen levels in mice derived from crosses between *Ptg*^{-/-} and *Gaa*^{-/-} animals. The glycogen levels of the *Gaa*^{+/+} *Ptg*^{+/+} (WT), *Gaa*^{+/+} *Ptg*^{-/-} (PKO), *Gaa*^{-/-} *Ptg*^{+/+} (GKO), and *Gaa*^{-/-} *Ptg*^{-/-} (GPKO) groups were 2.86±0.92, 1.37±0.30, 215.3±12.17, and 196.2±6.06 μmol glucose/gram tissue, respectively. Although the PKO group had more than 50% less glycogen content than the WT, the difference in values did not reach statistical significance (p = 0.19). This lack of statistical significance is likely due to a high SEM, and it is quite possible that increasing the number of animals might result in differences of statistical significance. The GKO group had approximately 75-fold greater glycogen content than the WT, and the difference was found to be of statistical significance (p = 1.2E-7). Similarly, the glycogen levels in the GPKO group were statistically significantly higher, by nearly 70-fold, than the WT (p = 1.1E-9). The GPKO was found to have slightly less glycogen than the GKO, though the difference was not statistically significant (p = 0.20).

Heart to body weight ratios (H/BW, see Figure 13) were used to determine the relative degree of cardiac hypertrophy among wildtype, single knockout, and double knockout mice for each group. The H/BW was calculated as milligrams of heart tissue per gram of body weight. Across all *Gaa*^{-/-} groups, high glycogen levels are strongly correlated with the development of cardiomegaly. In comparisons of H/BWs in the laforin group, there were no statistically significant differences between the *Gaa*^{+/+} *Epm2a*^{-/-} (LKO) and *Gaa*^{+/+} *Epm2a*^{+/+} (WT) groups (p = 0.53) or between the *Gaa*^{-/-} *Epm2a*^{-/-} (GLKO) and *Gaa*^{-/-} *Epm2a*^{+/+} (GKO) groups (p = 0.97). The H/BWs of both

the GKO and the GLKO groups were nearly 2-fold greater than the WT, and both of these differences were statistically significant ($p = 6.4E-4$ and $p = 2.0E-4$, respectively). Comparison of the H/BW of GLKO to the GKO groups did not result in a difference of statistical significance ($p = 0.97$).

In the malin group, it was found that the MKO group had a very slightly higher H/BW than the WT group, 4.0 versus 3.7, respectively, and although slight, this difference reached statistical significance ($p = 0.044$). Both the GKO and GMKO exhibited elevations in H/BW of statistical significance as compared to the WT; these elevations were 2-fold ($p = 7.7E-5$) and 1.8-fold ($p = 1.1E-4$), respectively. Although the H/BW of the GMKO group was lower than that of the GKO, the difference was not of statistical significance ($p = 0.38$).

Next, in the Stbd1 group, the SKO group had an approximately 10% lower H/BW than the WT group, but the reduction was not of statistical significance ($p = 0.29$). The H/BWs of both the GKO and GSKO groups were statistically significantly higher, about 2-fold, than that of the WT ($p = 3.1E-4$ and $p = 3.1E-5$, respectively). Comparison of the GSKO to the GKO group did not reveal a statistically significant difference in H/BW ($p = 0.60$).

Finally, in the PTG group, it was found that although the PKO group exhibited a nearly 10% reduction in H/BW as compared to the WT, the difference was not of statistical significance ($p = 0.24$). The GKO and GPKO groups demonstrated increases of 1.6-fold and 1.4-fold, respectively, in H/BW as compared to the WT group, and both of these differences were of statistical significance ($p = 4.8E-4$ and $p = 3.9E-3$, respectively).

Lastly, the GPKO group exhibited an approximately 10% reduction in H/BW as compared to the GKO group, but the difference was not statistically significant ($p = 0.17$).

Echocardiography (data not shown) was also performed. None of the analyses of the *Gaa*^{-/-} mice revealed significant defects in cardiac function, including cardiac output, ejection fraction, and fractional shortening, among other parameters. The primary difference of statistical significance detected in the *Gaa*^{-/-} mouse hearts as compared to the WT was increased posterior and anterior left ventricular wall thickness. Comparison of the *Gaa*^{-/-} single knockout groups (*Epm2a*^{+/+} *Gaa*^{-/-}, *Epm2b*^{+/+} *Gaa*^{-/-}, *Stbd1*^{+/+} *Gaa*^{-/-}, and *Ptg*^{+/+} *Gaa*^{-/-}) to the double knockout (DKO) groups, however, did not result in differences of statistical significance. These observations are in accordance with the H/BW results; the difference between the GKO and DKO groups was in no case found to be of statistical significance.

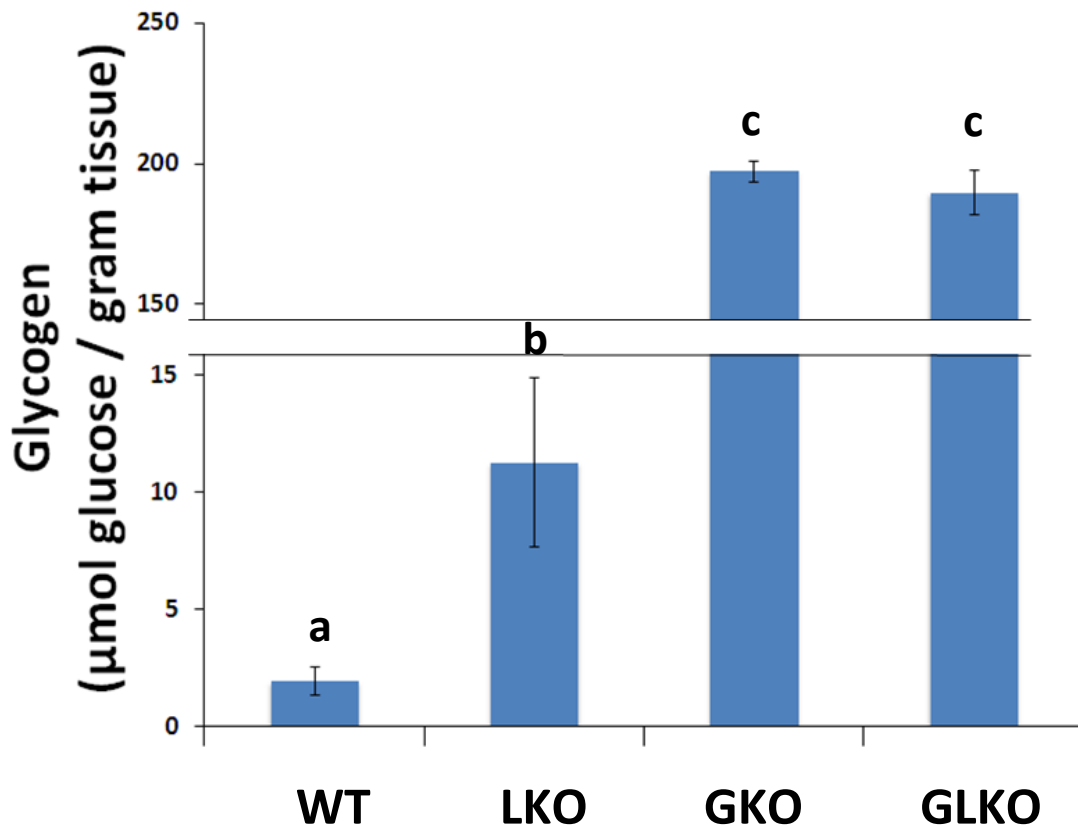


Figure 9. Glycogen in Heart Tissue from Mice Lacking Acid α -Glucosidase and Laforin. Glycogen levels in *Gaa*^{+/+} *Epm2a*^{+/+} (WT), *Gaa*^{+/+} *Epm2a*^{-/-} (LKO), *Gaa*^{-/-} *Epm2a*^{+/+} (GKO), and *Gaa*^{-/-} *Epm2a*^{-/-} (GLKO), were analyzed. Levels are expressed as glucose equivalents per gram of tissue. Values represent one assay for the GKO and GLKO groups and the average of two repeat assays for the WT and LKO groups \pm SEM. Each group consisted of 5 mouse hearts (n=5). Values marked by the same letter are not statistically significant; the different letters indicate $p < 0.05$.

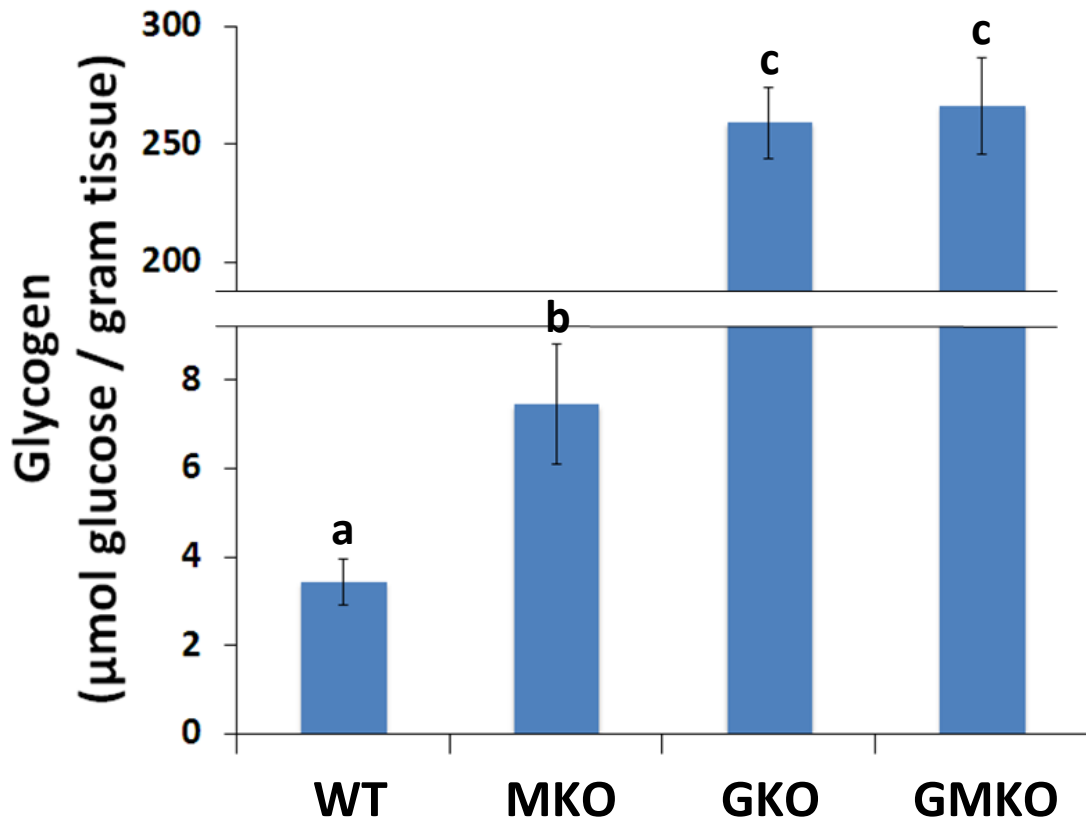


Figure 10. Glycogen in Heart Tissue from Mice Lacking Acid α -Glucosidase and Malin. Glycogen levels in *Gaa*^{+/+} *Epm2b*^{+/+} (WT), *Gaa*^{+/+} *Epm2b*^{-/-} (MKO), *Gaa*^{-/-} *Epm2b*^{+/+} (GKO), and *Gaa*^{-/-} *Epm2b*^{-/-} (GMKO), were analyzed. Levels are expressed as glucose equivalents per gram of tissue. Values represent one assay for the GKO and GMKO groups - and the average of two repeat assays for the WT and MKO groups \pm SEM. Each group consisted of 5 mouse hearts (n=5). Values marked by the same letter are not statistically significant; the different letters indicate $p < 0.05$.

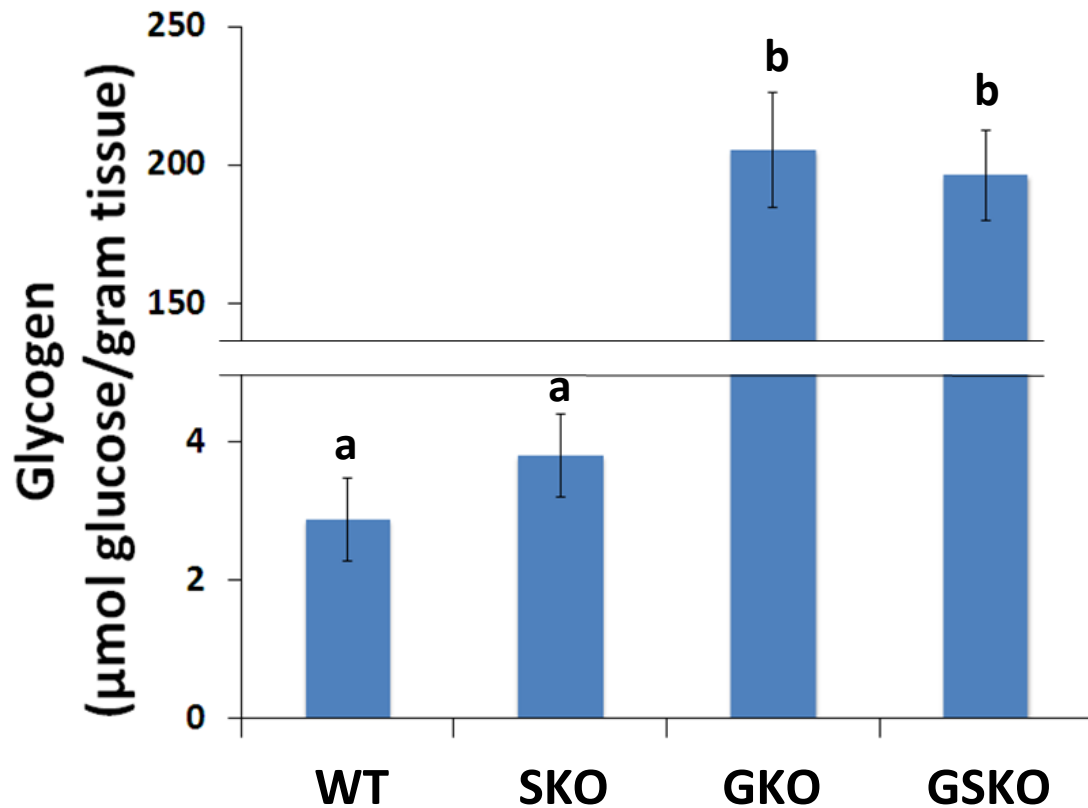


Figure 11. Glycogen in Heart Tissue from Mice Lacking Acid α -Glucosidase and Starch Binding Domain 1. Glycogen levels in *Gaa*^{+/+} *Stbd1*^{+/+} (WT), *Gaa*^{+/+} *Stbd1*^{-/-} (SKO), *Gaa*^{-/-} *Stbd1*^{+/+} (GKO), and *Gaa*^{-/-} *Stbd1*^{-/-} (GSKO), were analyzed. Levels are expressed as glucose equivalents per gram of tissue. Values represent one assay for the GKO and GSKO groups and the average of two repeat assays for the WT and SKO groups \pm SEM. Each group consisted of 5 mouse hearts (n=5). Values marked by the same letter are not statistically significant; the different letters indicate $p < 0.05$.

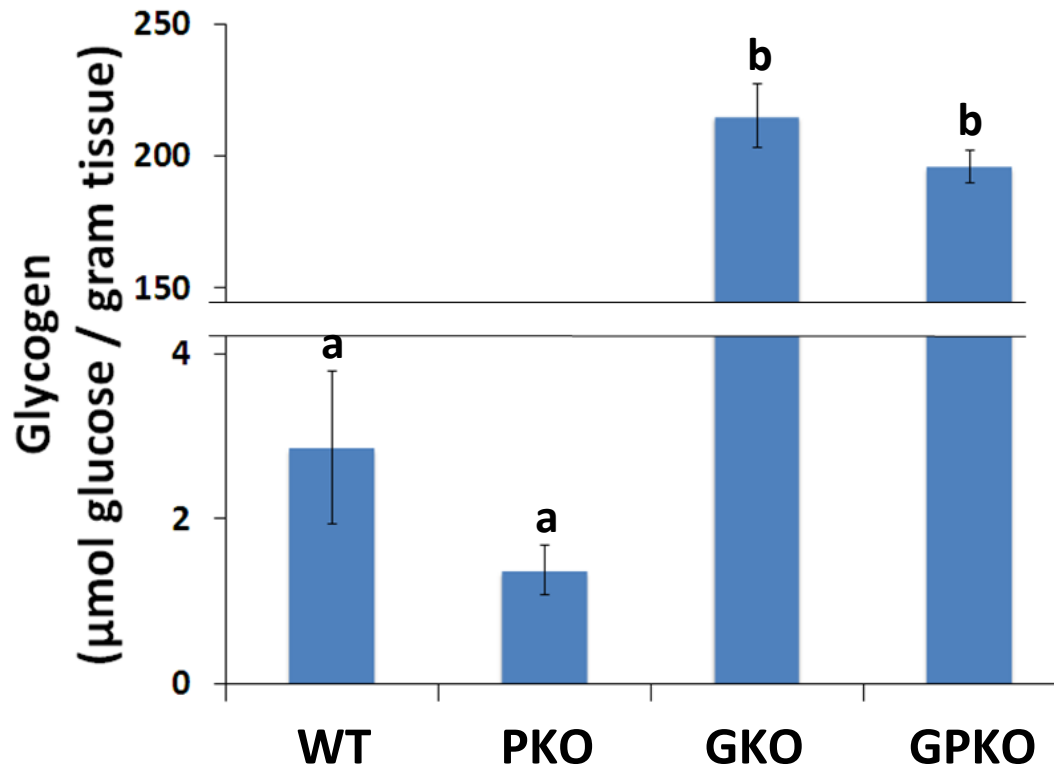


Figure 12. Glycogen in Heart Tissue from Mice Lacking Acid α -Glucosidase and Protein Targeting to Glycogen. Glycogen levels in *Gaa*^{+/+} *Ptg*^{+/+} (WT), *Gaa*^{+/+} *Ptg*^{-/-} (PKO), *Gaa*^{-/-} *Ptg*^{+/+} (GKO), and *Gaa*^{-/-} *Ptg*^{-/-} (GPKO), were analyzed. Levels are expressed as glucose equivalents per gram of tissue. Values represent one assay for the GKO and GPKO groups and the average of two repeat assays for the WT and PKO groups \pm SEM. Each group consisted of 5 mouse hearts (n=5). Values marked by the same letter are not statistically significant; the different letters indicate p<0.05.

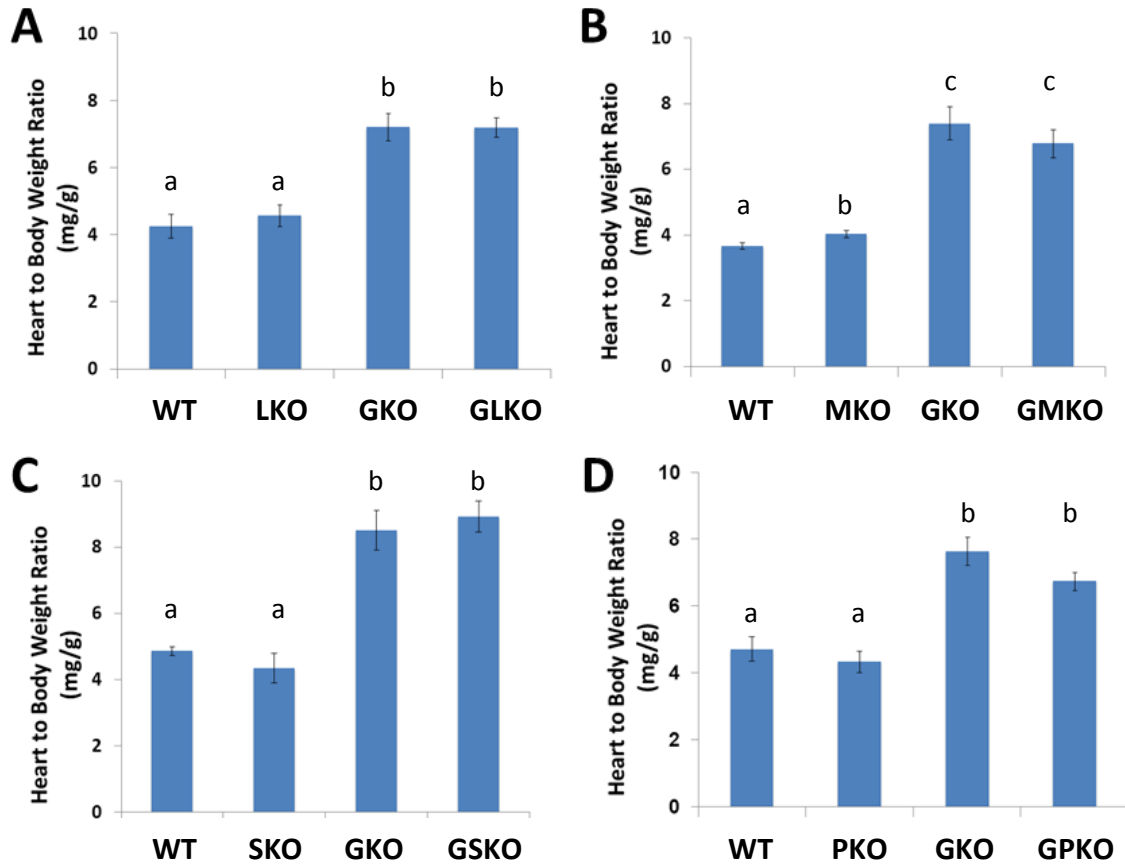


Figure 13. Heart to Body Weight Ratio in Mice Lacking Acid α -Glucosidase and Laforin, Malin, Starch Binding Domain 1, or Protein Targeting to Glycogen. Heart to body weight ratios were calculated and are expressed here as milligrams of heart tissue per gram of body weight. Values represent the average of five mouse hearts for each group, depicted \pm SEM. Values marked by the same letter are not statistically significant; the different letters indicate $p < 0.05$.

DISCUSSION

The hallmarks of Pompe disease, the substantial lysosomal glycogen accumulation and autophagic build-up, are reportedly the basis of the disease's pathologies (44) and likely also the cause of the fatal cardiomyopathy observed in severe, infantile PD cases (31,41). The aberrant lysosomal glycogen metabolism is the result of a genetic defect in acid α -glucosidase (GAA), the enzyme responsible for breaking down glycogen to glucose within the lysosomes (39). Although PD is an extremely rare disease, it can be fatal within the first year of life in severe cases and as such requires additional study and development of improved therapies. Pompe disease is currently being treated with enzyme replacement therapy (ERT), though unfortunately, the clinical success of ERT in PD patients has been seriously limited. Patients with severe infantile PD, who have received ERT, experience markedly improved cardiac function and lifespan (87,88) but at the cost of chronic, permanent disabilities, including devastating skeletal muscle myopathy, osteopenia, hearing loss, and severe gastroesophageal reflux (89-91). Recent success of substrate reduction therapy (SRT) in other lysosomal diseases (92) has stimulated interest in the possible application of SRT in Pompe disease treatment. Using primary myoblasts from *Gaa*^{-/-} mice, inhibition of the major glycogen synthesis enzyme, glycogen synthase (GYS1), resulted in reduced cytosolic and lysosomal glycogen levels, as well as reduced lysosomal size and number (93). Further studies with the *Gys*^{-/-} *Gaa*^{-/-} mice indicated normalized cardiac function, glycogen storage, autophagy, and exercise capacity (44). However, as GYS1 deficiency in results in a 90% death rate of mouse pups at birth due

to cardiac impairment (94), total GYS1 suppression may not be of utility in Pompe disease therapy. Partial inhibition, on the other hand, may yet be considered as possible a therapeutic approach.

In this study, we sought to characterize the effects of laforin, malin, Stbd1, and Ptg deficiencies on overall glycogen levels in heart tissue from *Gaa*^{-/-} mice in hopes of identifying a protein deficiency that may attenuate classic PD glycogen accumulation-linked cardiomyopathy. We postulated that the defects in autophagy observed in malin and laforin deficient mice, may consequently reduce lysosomal glycogen import and accumulation. However, neither *Gaa*^{-/-} *Epm2a*^{-/-} (GLKO) mice nor *Gaa*^{-/-} *Epm2b*^{-/-} (GMKO) mice exhibited significantly reduced glycogen levels in the heart tissue as compared to the single *Gaa*^{-/-} knockout (GKO) mice. Though both *Gaa*^{+/+} *Epm2a*^{-/-} (LKO) and *Gaa*^{+/+} *Epm2b*^{-/-} (MKO) mice exhibited significantly elevated glycogen levels as compared to the WT, this increase is likely in cytosolic glycogen rather than the lysosomal. While it is possible that laforin and malin do not affect how glycogen is trafficked to and accumulated within lysosomes in Pompe disease, these proteins may yet play some undetermined role, and should thus not be altogether ruled out.

Stbd1 deficiency was postulated to impair glycogen transport into lysosomes, thereby mitigating the primary pathology of Pompe disease. We did not, however, find a statistically significant reduction of glycogen levels in *Gaa*^{-/-} *Stbd1*^{-/-} (GSKO) mouse hearts as compared to *Gaa*^{-/-} *Stbd1*^{+/+} (GKO) hearts. These results are in accordance with another study, which conclude that *Stbd1* is not a probable candidate for Pompe therapy research (79).

Lastly, *Ptg*^{-/-} mice were analyzed, as previously PTG deficiency in both *Epm2a*^{-/-} and *Epm2b*^{-/-} mice resulted in significant reduction of glycogen levels as well as abrogation of both Lafora bodies and neurological disorders. Our rationale was that a decrease in glycogen synthesis may also reduce lysosomal accumulation of glycogen in *Gaa*^{-/-} mice. In this study however, we did not detect a statistically significant decrease in the glycogen levels of the *Gaa*^{-/-} *Ptg*^{-/-} (GPKO) hearts as compared to the *Gaa*^{-/-} *Ptg*^{+/+} (GKO) hearts.

In conclusion, analyses of *Epm2a*^{-/-}, *Epm2b*^{-/-}, *Stbd1*^{-/-}, and *Ptg*^{-/-} in a *Gaa*^{-/-} mouse model background did not reveal reductions of statistical significance in either heart tissue glycogen levels or cardiac hypertrophy. However, in the laforin and malin groups, the sum glycogen content of the LKO or MKO and GKO groups was not obviously equal to that of the DKO groups. Inability to detect additivity between these groups may be due to the standard error. Another possibility is that these mouse heart cell have simply reached maximum glycogen capacity; the *Gaa*^{-/-} hearts had over 100-fold higher glycogen levels than the WT. Alternatively, as *Epm2a*^{-/-} and *Epm2b*^{-/-} mice exhibit elevated levels of cytosolic glycogen, it is possible that lysosomal glycogen content was reduced in the GLKO and GMKO groups; however, the elevation of cytosolic glycogen compensated for the reduction of lysosomal glycogen in GLKO and GMKO hearts, thereby resulting in the lack of additivity. In this study, we quantified total glycogen levels and did not differentiate between lysosomal and cytosolic glycogen. In the future, these issues could be addressed by isolation of the lysosomes to specifically quantify glycogen levels therein, or by histological analyses of heart tissue sections with

periodic acid–Schiff (PAS) staining to determine the localization of the glycogen accumulation.

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